ROLE OF FIP200 IN CEREBELLAR DEGENERATION

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Chun-Chi Liang

August 2009

ROLE OF FIP200 IN CEREBELLAR DEGNERATION

Chun-Chi Liang, Ph. D.

Cornell University 2009

Although the primary function of autophagy is the supply of amino acids as a response to starvation conditions in many organisms, recent studies suggested that basal autophagy, which occurs independently of nutrient stress, also plays an important role in maintaining cellular homeostasis, particularly in quiescent cells such as neurons. FIP200 (FAK family interacting protein of 200 KDa) was recently proposed as a mammalian counterpart of yeast Atg17 (autophagy-related 17) required for autophagosome initiation despite its different structure and sequence from Atg17. However, it remains unclear whether mammalian FIP200 regulates autophagy *in vivo* and the regulation is also involved in neuronal homeostasis and neurodegeneration. In this study, the results collected from the brain of which FIP200 was depleted by nestin-Cre showed that cerebellar degeneration and ataxia accompanied by progressive loss of Purkinje cells, swelling and degeneration of their axons, and spongiform degeneration in the cerebellum. Further analyses by conditional deletion of FIP200 using hGFAP-Cre and L7-Cre with complementary Cre expression in the cerebellum provided support for a Purkinje cell-autonomous function of FIP200 in the regulation of cerebellar degeneration. Consistent with a role of FIP200 in autophagy, observed progressive accumulation of abnormal ubiquitinated protein aggregates without any impairment of the ubiquitin-proteasome activity, increased

apoptosis, and mitochondrial damage in Purkinje cells were found in the mutant mice. Lastly, the study demonstrated that deletion of TNF-R1 rescued both the loss of Purkinje cells and spongiform degeneration in FIP200 conditional KO mice. Together, these results provided compelling genetic evidence that FIP200 regulation of autophagy and TNFR1 signaling playscritical roles in the pathogenesis of neurodegenerative disorders in mammals.

BIOGRAPHICAL SKETCH

Chun-Chi Liang attended National Taiwan University to study Agricultural Chemistry and graduated with the Presidential Award in 1997. After couple years of his military service, he turned to laboratory research working with Dr. Hong-Chen Chen at National Chung Hsing University. His work there revealed that hepatocyte-growth factor can trigger sustained-activation of extracellular signal-regulated kinases, leading to a significant increase in the expression of integrin alpha2 as well as the cell motility. Aside from the study of cell motility, he also discovered a synergistic effect of hepatocyte-growth-factor and Focal-Adhesion-Kinase on the induction of epithelial-mesenchymal-transition and its role in cell transformation. He then entered the Ph.D. Program in Comparative Biomedical Sciences at Cornell University and joined the laboratory of Dr. Jun-Lin Guan to continue his investigation on the molecular mechanisms of cell motility as well as on the neuroscience aspects of FIP200 transgenic model in 2002. His research projects mainly focuses on the FIP200 regulates cell-autonomous neurodegeneration and embyogenesis. By using *in vivo* genetic approaches, he successfully revealed that FIP200 is a critical molecule involved in TNF-R1 signaling pathway to maintain homeostasis and survival of neuronal cells. Following award of her doctoral degree in April 2009, he continues his interest in the role of FIP200 in neural stem cell and neurogenesis in Dr. Jun-Lin Guan's laboratory at University of Michigan. Currently, he is also an active member of the Society for Neuroscience.

To my mother, sister, and brother

for always being there for me

ACKNOWLEDGMENTS

The research and writing of a dissertation can be a lonely and isolating experience, yet it is obviously not possible without the personal and practical support of many people. Thus my sincere gratitude goes to my parents and all my friends for their love, support, and patience over the last several years.

This dissertation would not have been possible without the expert guidance of my esteemed advisor, Prof. Jun-Lin Guan. This is a great opportunity to express my respect to him for accepting me as his graduate student and his immeasurable training, advice and encouragement. Not only has he taught me a variety of cell biology and biochemistry techniques, but he has helped me to prepare for my future career in many other ways. He trained me to be an independent researcher and gave me courage to aim high in my future career.

I am pleased to thank the members of my special committee, Drs. Richard A. Cerione, Anthony P. Bretscher, Michael I. Kotlikoff, and Robert S. Weiss for being there when I was in need of ideas and suggestions. Many people on the faculty and staff of the University of Michigan assisted and encouraged me in various ways during my course of studies. I am especially grateful to Drs. Henry Paulson, Yuan Zhu, Andy Lieberman of University of Michigan for discussions, reagents, help throughout this project, and critical reading of the manuscript and helpful comments. I also wish to thank Dr. Geoffrey Murphy of University of Michigan for help in Rota-rod tests and Dr. Hisashi Umemori of university of Michigan for assistance in Purkinje cell culture.

My graduate studies would not have been the same without the social and academic challenges and diversions provided by all my student-colleagues in the Department of Molecular Medicine. I am particularly thankful to my friend, Joseph E. Druso. Without his friendship and help, it would not have been possible that I could handle the animal studies so quickly and easily. I also thank past and present members of Guan laboratory for their friendship, critical reading of the manuscript, suggestions and technical assistance.

I wish to acknowledge both the National Institutes of Health for providing funding to Dr. Jun-Lin Guan.

My enormous debt of gratitude can hardly be repaid to my family. I am grateful to my mother, sister, and brother for their love, support, and understanding.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1
INTRODUCTION

1.1 FIP200

FIP200 (FAK-family Interacting Protein of 200 kDa) was first characterized as a Pyk2 interacting protein through a yeast two-hybrid screen in our laboratory in 2000 (Ueda et al., 2000). Before our characterization, the full length cDNA for FIP200 (named as KIAA0203) was already isolated in projects of sequencing human cDNA clones (Nagase et al., 1996), but without any details or further describing of its possible functions. In the late-90's, Maucuer found that fragments of FIP200 (designated as CC1) functions as a stathmin interacting protein (Maucuer et al., 1995). Soon after Maucuer's report, Fragments of FIP200 (named as LaXp180) were also found as a binding partner of the Listeria monocytogenes surface protein ActA in Pfeuffer's study (Pfeuffer et al., 2000). In 2002, another independent identification for full length FIP200 reported it as a potential regulator of the RB1 gene (designated as RB1CC1 for RB1-inducible coiled-coil 1) (Chano et al., 2002b). Further studies have shown that FIP200 functions as a critical signaling molecule to regulate cell growth, cell proliferation, cell survival, and cell spreading/ migration. Furthermore, deletion of FIP200 has been shown to lead to embryonic lethality in mice (Gan et al., 2006).

1.1.1 FIP200 gene expression, protein structure and subcellular localization

FIP200 is an evolutionarily conserved protein in human, mouse, rat, Xenopus laevis, Drosophila melanogaster and Caenorhabditis elegans. Although the function of FIP200 orthologues in other species remains unknown, studies have shown that FIP200 is widely expressed in various

human and mouse tissues, particularly abundant in heart, testis and musculoskeletal systems (Chano et al., 2002b; Chano et al., 2002d). More interestingly, FIP200 is copiously expressed throughout mouse embryonic development (Bamba et al., 2004), suggesting that FIP200 may play an important role in embryogenesis.

The Mouse FIP200 gene localizes in chromosome 1A2-4, which is syntenic to human chromosome 8q11. Both mouse and human FIP200 genes consist of 24 exons and share similar genomic structures. In addition, FIP200 proteins in mouse and human share around 90% identity and similar domain structure (Chano et al., 2002b). FIP200 gene encodes a 200 kDa protein (1591 aa) with several identifiable domains, such as a large coiled-coil region (residues 860–1391), a leucine zipper motif (residues 1371–1391), and a putative nuclear localization signal (NLS). (Figure 1.1) In summary, FIP200 has been mostly reported as a cytoplasmic protein (Gan et al., 2005; Gan et al., 2006; Maucuer et al., 1995; Pfeuffer et al., 2000; Ueda et al., 2000) and conditionally localizes in focal adhesion (Abbi et al., 2002) as well as nucleus (Chano et al., 2002b; Melkoumian et al., 2005).

The studies in first decade have identified 10 proteins as FIP200 interacting proteins, including stathmin (Maucuer et al., 1995), Pyk2 (Ueda et al., 2000), FAK (Abbi et al., 2002), ActA (Pfeuffer et al., 2000), p53 (Melkoumian et al., 2005), TSC1 (Chano et al., 2006; Gan et al., 2005), ASK1, TRAF2 (Gan et al., 2006), ULK1 (Hara et al., 2008), and Atg13 (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009). Because these interacting proteins regulate different cellular functions including microtubule dynamics, adhesion, migration, proliferation, growth, and stress response, the data suggest that FIP200 may play very diverse biochemical functions with different binding

Figure 1.1 | Structural domains of FIP200.

FIP200 consists of a putative nuclear localization signal (NLS) at N-terminus, a large coiled-coil (CC) domain and a leucine zipper (LZ) motif located at C-terminus.

partners to integrate various signaling pathways in cells (Figure 1.2).

1.1.2 FIP200 function in cell survival

As mentioned earlier, FIP200 was initially identified as a Pyk2 interacting protein (Ueda et al., 2000) and their interaction was shown to inhibit the kinase activity of Pyk2, which can suppress Pyk2-induced apoptosis (Avraham et al., 2000; Xiong and Parsons, 1997). Our study also showed that activation of Pyk2 correlates with dissociation of endogenous FIP200–Pyk2 interaction under several biological stimuli. Taken collectively, it indicates that FIP200 functions to promote cell survival through its inhibition of Pyk2 activity.

Importantly, the pro-survival function of FIP200 was further investigated in recent FIP200 knockout (KO) mice study. The study has shown that homozygous deletion of FIP200 in mouse leads to embryonic lethality associated with massive apoptosis in liver and heart (Gan et al., 2006). To further understand the mechanism of regulation of apoptosis by FIP200, FIP200 KO MEFs and fetal liver cells have been isolated; however, these cells do not show increased apoptosis either under normal or various apoptosis-induced culture conditions, such as energy deprivation or sorbitol treatment. Interestingly, FIP200 KO MEFs and fetal liver cells show an elevated sensitivity to TNFα-induced apoptosis. It has been known that TNFα activates JNK through TNFR–TRAF2–ASK1–MKK4/MKK7–JNK signaling cascade (Davis, 2000; Lee et al., 1997; Nishitoh et al., 1998; Tobiume et al., 2001). Our further studies show that FIP200 associates with ASK1 and TRAF2, functions as a scaffold protein to orchestrate TRAF2–ASK1 signaling. The protein complexes are capable of increasing JNK serine/threonine-

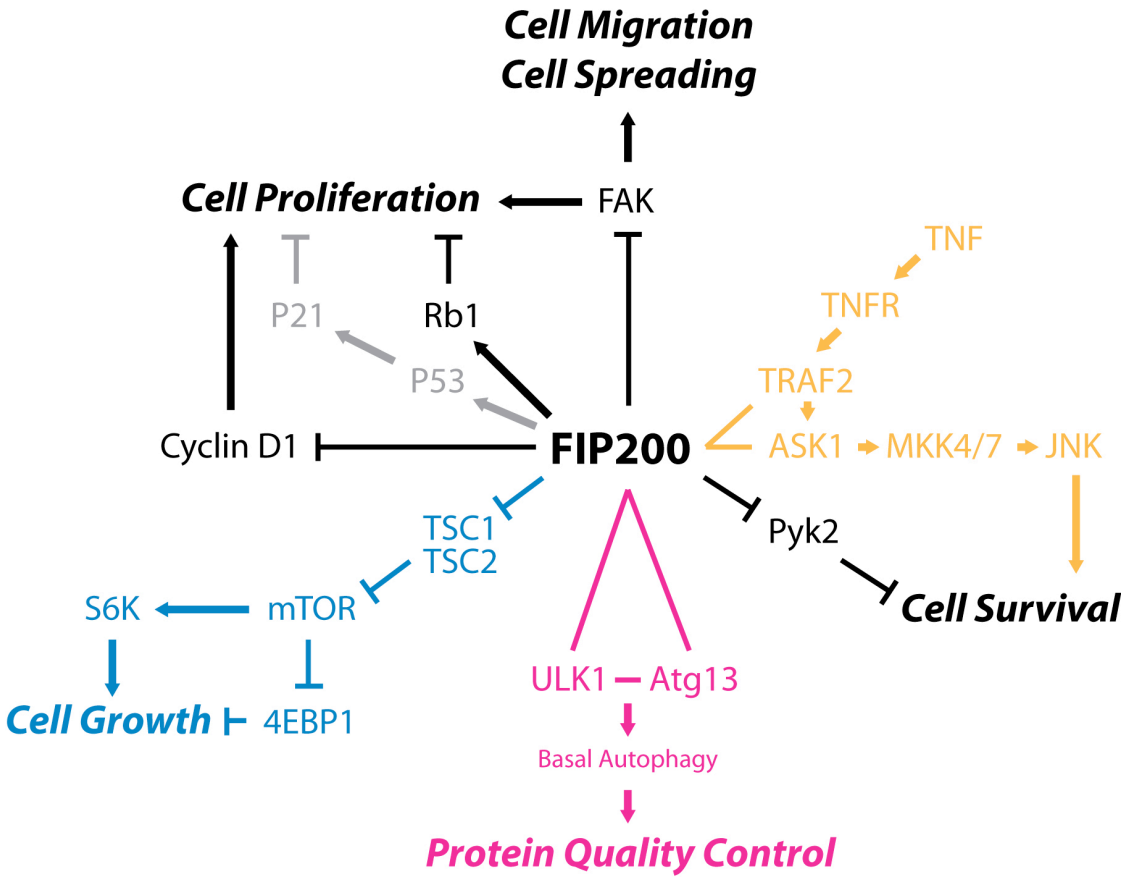


Figure 1.2 | FIP200 signaling pathway.

FIP200 functions to regulate diverse cellular processes, including cell proliferation, cell spreading, cell migration, cell growth, cell survival and protein quality control. FIP200 suppresses cell proliferation by regulation of p53–p21, cyclin D1, Rb1 and FAK pathways; FIP200 negatively regulates cell spreading and migration by its inhibition of FAK function; FIP200 promotes mTOR activation and cell growth through its interaction with TSC1–TSC2 complex; FIP200 positively regulates cell survival by its inhibition of Pyk2 activity and regulation of TNFα–JNK signaling cascade; FIP200 initiates basal autophagosome formation to remove aberrant protein aggregation by forming a protein complex with ULK1 and Atg13.

phosphorylation upon TNFα stimulation (Gan et al., 2006). Because NF-κB signaling and Pyk2 activation remain unaffected in FIP200 KO MEFs and liver cells, the defective JNK pathway is subsequently identified to be responsible for increased TNFα-stimulated apoptosis (Gan et al., 2006). In summary, FIP200 promotes cell survival through at least two different mechanisms. One is through inhibition of Pyk2 activity; the other is through TNFα-JNK-dependent mechanism.

1.1.3 FIP200 function in cell proliferation

Our lab initially showed that over-expression of FIP200 inhibits cell proliferation in NIH3T3 cells and the inhibition of cell proliferation could be reversed by co-expression of FAK. Furthermore, we found that the disruption of the interaction between endogenous FIP200 and FAK leads to enhanced tyrosine phosphorylation of FAK and partial restoration of cell cycle progression of cells plated on poly-L-lysine (Abbi et al., 2002). These results suggest that FIP200-mediated inhibition of cell proliferation is at least partly through its inhibition of FAK signaling. Another study from our lab documents that over-expression of FIP200 can also inhibit cell proliferation and induce cell cycle G1 phase arrest in human breast cancer cells (Melkoumian et al., 2005) through an increase of p21 and a decrease of cyclin D1 protein levels. The upregulated p21 expression is because of stabilized p53 in nucleus by the interaction of FIP200 with p53. In contrast to p21, the decrease of cyclin D1 protein by FIP200 is facilitated by an increased proteasome-dependent degradation of cyclin D1. Interestingly, it is found that FIP200-induced G1 arrest is independent of FAK pathway in the breast cancer cells studied (Melkoumian et al., 2005). In other words, FIP200 can regulate

cell proliferation through FAK dependent and independent mechanisms.

Many studies have suggested that expression or tyrosine-phosphorylation levels of FAK correlate with cancer development (Agochiya et al., 1999; Jones et al., 2000; Owens et al., 1995). Conditional deletion of FAK in mice suppresses chemically induces skin tumor formation and blocks malignant progression (McLean et al., 2004), providing the direct evidence implicating the critical role of FAK in tumorigenesis. These studies, therefore, raise an interesting possibility that FIP200 might potentially function as a tumor suppressor through the inhibition of FAK activity and cell proliferation. Besides, over-expression of FIP200 has been shown to inhibit cell proliferation resulting from upregulated expression of RB1 in human leukemic cells, such as K562 and Jurkat cells (Kontani et al., 2003). RB1 expression correlates with truncated FIP200 expression in the breast cancer cells. These data suggest that FIP200 regulation of RB1 expression might play a role in its tumor suppression (Chano et al., 2002c).

Therefore, these studies provide strong evidence that FIP200 functions as a negative regulator in cell cycle progression through different downstream signaling pathways in different cellular contexts.

1.1.4 FIP200 function in protein synthesis and cell growth

Cell growth (increase in cell mass and size) is an orchestrated cellular process, which must be tightly regulated by both intracellular and extracellular stimuli. The studies from the last decade have identified the mammalian target of rapamycin (mTOR) as a critical regulator of cell growth through its regulation of a variety of cellular functions, including initiation of mRNA translation, ribosome synthesis, expression of metabolism-related

genes and autophagy (Schmelzle and Hall, 2000). The mTOR activity can be up-regulated by the small GTPase Rheb (Ras homolog enriched in brain) or down-regulated by TSC1/2 complex, which stimulates Rheb GTP hydrolysis. Therefore, phosphorylation levels of ribosomal S6 kinase (S6K) and eukaryotic initiation factor 4E binding protein-1 (4EBP-1) (Wullschleger et al., 2006), two key downstream targets of mTOR, are regulated by growth factors and nutrient stimuli.

Recently, FIP200 was identified as a TSC1 interacting protein through yeast two-hybrid screens (Gan et al., 2005). Our study showed that the interaction of FIP200 and TSC1 correlates with mTOR activation and cell growth. Over-Expression of FIP200 up-regulates mTOR activation and increases cell size whereas RNAi knockdown of endogenous FIP200 suppresses mTOR activation and reduces cell size (Gan et al., 2005). FIP200 also has been shown to inhibit TSC1-TSC2 complex by promoting TSC1 degradation through the ubiquitin proteasome pathway (Chano et al., 2006). Taken collectively, the results suggest that FIP200 inhibits TSC function through disruption of TSC1–TSC2 complex formation, therefore increasing activity of mTOR.

Moreover, over-expression of FIP200 can promote mTOR activation and cell growth under nutrient deprived conditions. It has also been documented that FIP200 is important in nutrient stimulation-induced, but not energy- or serum-induced, mTOR activation (Gan et al., 2005). Interestingly, FIP200 is independently identified as the regulator of TSC-mTOR signaling by a different approach as well (Chano et al., 2006). These studies show that RNAi knockdown of FIP200 in mouse muscle cells reduces cell size, suggesting that

FIP200 may play a role in the regulation of muscle hypertrophy/atrophy *in vivo* (Chano et al., 2006).

In summary, FIP200 functions as a promoter in protein synthesis and cell growth by inhibiting the function of tumor suppressor TSC1 and TSC2, activating mTOR signaling.

1.1.5 FIP200 function in autophagy

Recently FIP200 has been identified as a novel ULK1-interacting protein. The interaction of FIP200 and ULK1 is via the C-terminal region of ULK1 (Hara et al., 2008). In addition, FIP200 is identified as a substrate of ULKs. The phosphorylation of FIP200 by ULK1 can be detected in 293T lysates over-expressing FIP200 and ULK1 (Ganley et al., 2009) and at autophagosomal structures under mTOR inactivation (Hara et al., 2008). Because FIP200 KO MEFs have no detectable autophagosome (Hara et al., 2008), the results suggest that the interaction of FIP200 and ULK1 plays a role in an early step of autophagosome formation. Recent studies have shown that FIP200 is a part of the ULK1-Atg13-FIP200 protein complex (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009) and the complex is prior to autophagy induction (Ganley et al., 2009). Interestingly, aside from its interaction with Atg13 (Chan et al., 2009) and FIP200 (Hara et al., 2008), ULK1 can also bind to SynGAP and syntennin to regulating the neuronal axon outgrowth (Chan et al., 2007; Tomoda et al., 2004). The results suggest that FIP200 may have important functions in neurons.

1.1.6 FIP200 function in cell spreading and migration

FIP200 has been shown to colocalize with FAK at focal adhesions through its association with FAK kinase domain. The interaction between FIP200 and FAK inhibits FAK kinase activity, autophosphorylation of tyrosine-397, and cellular functions in cell spreading and migration (Abbi et al., 2002). The endogenous interaction between FIP200 and FAK is detected in suspended cells but decreased upon cell attachment on fibronectin (FN) (Abbi et al., 2002), suggesting that integrin signaling negatively regulates this interaction to activate FAK and its downstream pathways. Additionally, co-expression of FAK can reverse the inhibition of cell spreading and migration by FIP200, suggesting that FIP200 regulation of cell spreading and migration is through its inhibition of FAK signaling.

Cell spreading and migration play essential roles in numerous biological processes such as embryogenesis, angiogenesis, wound repair, inflammatory immune response, and cancer metastasis (Christopher and Guan, 2000; Lauffenburger and Horwitz, 1996). Studies have reported that FAK functions as a crucial molecule in integrin-mediated cell spreading and migration (Abbi and Guan, 2002; Mitra et al., 2005). Other studies have also identified several protein tyrosine phosphatases, such as PTEN, as a FAK inhibitor by tyrosine-dephosphorylation of FAK (Tamura et al., 1998; Yu et al., 1998). In contrast, FIP200 binds to the FAK kinase domain to inhibite FAK kinase activity, representing an alternative regulatory mechanism of FAK function. Since FAK activity has been implicated in cancer metastasis (Mitra and Schlaepfer, 2006), generation of small peptides or their derivatives from FIP200 as inhibitors for FAK could be of potential therapeutic significance.

1.1.7 FIP200 function in cell differentiation

The potential role of FIP200 in cell differentiation is first implicated by its increasing expression during the maturation process of musculoskeletal cells (Chano et al., 2002a). Another example, upon myoblast differentiation FIP200 gene expression is induced in C2C12 cells, it correlates with the upregulation of Rb1 and myoblast differentiation marker proteins Myhc (Watanabe et al., 2005). The C2C12 myoblast differentiation process can be suppressed by RNAi knockdown of endogenous FIP200 (Watanabe et al., 2005). Together, these data identify that FIP200 as a potential regulator of myogenic differentiation.

1.1.8 The role of FIP200 in embryonic development

FIP200 is copiously expressed throughout mouse embryonic development (Bamba et al., 2004). The expression pattern suggests that FIP200 may play an important role in embryogenesis. Our study has shown that targeted ablation of FIP200 in mice leads to embryonic lethality at mid/late gestation associated with heart failure and liver degeneration (Gan et al., 2006). FIP200 KO embryos at E14.5 and E15.5 lose much of the normal trabecular and external compact myocytes, resulting in significantly thinner heart ventricular walls with fewer cells. Besides, the majority of the FIP200 KO embryos also show severe liver degeneration characterized by loosely arranged hepatocytes mixed with numerous red blood cells. Hepatocytes are isolated from each other because of hemorrhage in the liver. Our study further identifies that the heart failure and liver degeneration found in FIP200 KO embryos results from massively increased apoptosis (Gan et al., 2006).

FIP200 has been shown to regulate cell size through its interaction with

TSC1–TSC2 complex. Interestingly, TSC1 and TSC2 KO embryos present severe heart defects with thickened ventricular walls (Kobayashi et al., 1999; Kobayashi et al., 2001). The opposite defective cardiac phenotypes suggest that FIP200 might function as the antagonist for TSC1-TSC2 complex. In addition, the isolated cardiomyocytes from FIP200 KO embryos show decreased mTOR activation and reduced cell size (Gan et al., 2006). Taken together, the *in vivo* results indicate that FIP200 play an important role in cell size/cell growth during heart development.

The liver degeneration phenotype found in FIP200 KO embryos is similar to the KO embryo phenotypes of several deletions of components of TNFα signal pathways, including Rel A, IKK-β, IKK-γ, GSK-3, MKK4, MKK7, or c-Jun. These embryos are characterized by mid/late gestational lethality associated with increased apoptosis in liver as well (Beg et al., 1995; Hilberg et al., 1993; Hoeflich et al., 2000; Li et al., 1999; Nishina et al., 1999; Rudolph et al., 2000; Wada et al., 2004). Furthermore, FIP200 KO MEFs exhibit increased apoptosis upon TNFα treatment because of reduction of JNK phosphorylation. Additionally, analysis shows that the interaction of FIP200 with TRAF2 and ASK1 is responsible for regulating ASK1 and JNK phosphorylation for cell survival (Gan et al., 2006).

1.1.9 The role of FIP200 in psoriasis

Using a conditional KO approach, ablation of FIP200 in skin causes skin inflammatory diseases resembling aspects of human psoriasis (Wei et al., 2009). The mutant mice showed activation of NF-ϰB and up-regulation of proinflammatory cytokines, including TNFα, IL-1β, IL-6, and IFNγ in the skin environment. These proinfnflammatory cytokines are also seen in lesions

of human psoriasis. Although previous studies suggest that inactivation of FIP200 does not affect NF-κB signaling in MEFs (Gan et al., 2006), ablation of FIP200 in keratinocytes could interfere with the balance of NF-κB activity to trigger an inflammatory response.

1.1.10 The potential Role of FIP200 in Alzheimer's disease (AD)

FIP200 is copiously expressed in both developing and mature neuronal cells. A recent study suggests that FIP200 may play a role in the regulation of neuronal size through its regulation of TSC1 degradation, mTOR, and RB1 (Chano et al., 2006). Analysis of samples from AD patients showed that 31% of the samples exhibited a decrease in FIP200 expression and phosphorylation of S6. These studies also demonstrate that specific knockdown of FIP200 in Neuro-2a cells induces the inhibition of the mTOR signaling pathway, resulting in neurite atrophy and cell death. Thus, these observations suggest that insufficiency or dysfunction of FIP200 may be involved in the pathogenesis of AD or contributes to the pathogenesis of neurodegenerative brains like AD (Chano et al., 2007).

1.2 Cerebellum

Despite its small portion to total volume of the brain in mice, the cerebellum contains more than half of all the neurons in the central nervous system (CNS) (Kandel et al., 2000). These neurons are arranged in a highly regular order of cellular organization resulting from repetition of the same neural circuit module. The structural regularity of the cerebellum suggests that all neural circuit modules execute similar functions. Interestingly, the cerebellum can be further dissected into several distinct regions. Each region

of the cerebellum functions on a distinct set of inputs and connects with different areas of the brain. Thus, the cerebellum indirectly mediates movement and posture by modifying the output of the major descending motor systems in CNS (Ito, 1984). Different from the lesions of the motor cortex, which reduce the movement strength and speed resulting from losing the capability of individual muscles to contract, damages occurred in the cerebellum distort movement coordination of limb and eye, cause an impairment of balance, and decrease muscle tone.

1.2.1 The developmental organization of the cerebellum

The spatial organization of the cerebellum is crucial for its proper functioning. Such a structure is the result of the subtle modification from the differential neuronal proliferation, selective cell death, and cell migration during development. The mouse cerebellum reaches its final configuration over a 6-week period between embryonic age E7 and postnatal age P30. The origin of all cerebellar neuronal precursors is a germinal matrix consisting of the ventricular neuroepithelium and the rhombic lip (Goldowitz and Hamre, 1998).

The interneurons in the cerebellar cortex arise from the metencephalon and the mesencephalon (Manto and Pandolfo, 2002). The ventricular neuroepithelium produce cerebellar nuclear neuroblasts, Purkinje neuroblasts, and Golgi neuroblasts (Goldowitz and Hamre, 1998; Wang and Zoghbi, 2001). The generation of cerebellar nuclear neuroblasts from the ventricular epithelium is further characterized as the first migration stage of primitive cells. Initial cluster of Purkinje neuroblasts moves outward radially during the second migration stage. Granule cell neuroblasts, basket cell neuroblasts and

stellate cell neuroblasts migrate away from germinal neuroephithelium to the outer surface of the developing cerebellum, forming the external granule layer. The outer boundary of the external granule layer is composed of proliferating neuroblasts but the inner compartment contains post-mitotic precursors of granule neurons. Interestingly, the size of the population of granule neurons is determined by the number of Purkinje cells during the development of the cerebellum. This is that the Purkinje cell secretes Sonic hedgehog to sustain the mitotic activity of granule neuron precursors within the external granule layer (Wallace, 1999). Then, these post-mitotic granule neurons migrate into the developing cerebellar white matter to generate the internal granule layer, which is characterized as the third migration stage. During this final migration stage, the neural-glial interaction between granule cell precursors and Bergmann glia is important for positioning granule neurons. The granule cell precursors migrate via the long processes of the Bergmann glia (Rakic and Sidman, 1973) till reaching their destination. During the third migration stage, the external granular layer will disappear progressively (Altman, 1972a; Altman, 1972b; Rakic, 1971).

1.2.2 The composition of cerebellum

The cerebellum has a unique structure to wrap up an enormous number of interconnected elements into a small volume (Figure 1.3). It is composed of the cerebellar cortex and a set of cerebellar nuclei. The morphological unit of the cerebellum is a folium, which contains afferents and efferents in the middle and the cerebellar cortex on both sides. Interestingly, the cerebellar cortex contains more neurons and synapses than the rest of the brain. The cerebellar cortex is a simple but highly regular structure which only

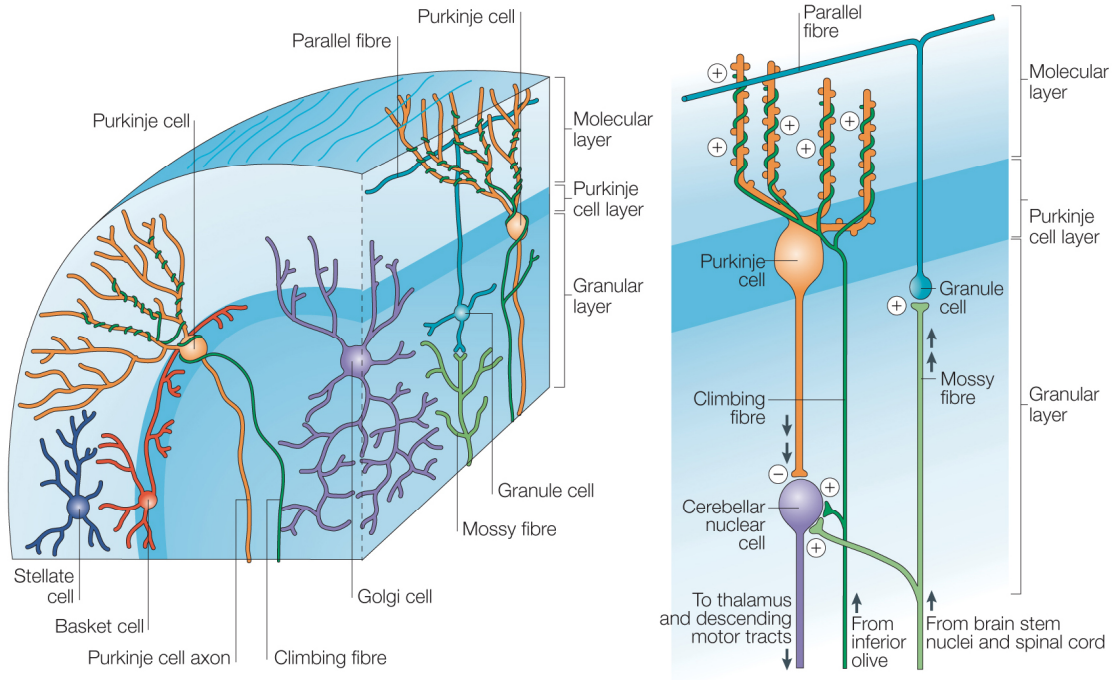


Figure 1.3 | Basic structure of the cerebellar cortex.

There are two main afferents to the cerebellar cortex: climbing fibres, which make direct excitatory contact with the Purkinje cells, and mossy fibres, which terminate in the granular layer and make excitatory synaptic contacts mainly with granule cells, but also with Golgi cells. In some cases, the stem axons of climbing and mossy fibres also provide collaterals to the cerebellar nuclei en route to the cerebellar cortex. The ascending axons of the granule cells branch in a T-shaped manner to form the parallel fibres, which, in turn, make excitatory synaptic contacts with Purkinje cells and molecular layer interneurons — that is, stellate cells and basket cells. Typically, parallel fibres extend for several millimetres along the length of individual cerebellar folia. With the exception of granule cells, all cerebellar cortical neurons, including the Purkinje cells, make inhibitory synaptic connections with their target neurons †From (Apps and Garwicz, 2005), courtesy of the author.

consists of three distinct layers-molecular layer, Purkinje cell layer, and granule layer (Kandel et al., 2000)-as well as eight types of neurons-basket cells, stellate cells, Purkinje cells, Lugaro cells , Golgi cells, granule cells, unipolar brush cells and candelabrum cells (Laine and Axelrad, 1994; Mugnaini and Floris, 1994).

1.2.2.1 Granule Cells

The innermost granular layer contains a huge number of tightly packed small granule cells as well as two inhibitory interneurons, Lugaro cells and Golgi cells. Granule cells are round or oval shape, with a diameter of 5-8 μm, and are the main component of the granular layer. In humans, the number between 10^{10} and 10^{11}, with about 3 to 7 million cells/mm^3, has been estimated as the total amount of granule cells. Each granule cell has thinness of the rimming cytoplasm and presents about 4 to 5 dendrites, forming excitatory synaptic contacts with the mossy fiber branches at the cerebellar glomeruli. The granule dendrites also construct inhibitory synapses with axons of Golgi cells. The axon of the granule cells is unmyelinated and extends up perpendicularly to the molecular layer, where it forms T-shaped branches running parallel to the axis of the folium in opposite directions. These parallel fibers form excitatory synapses with the spines of the Purkinje cell dendritic arbor. Approximately, one Purkinje dendritic tree has 4,000,000 parallel fibers cross through and about 80,000 parallel fibers form a single synapse with the same number spines of one Purkinje cell. Primarily, these parallel fibers consist of the outermost molecular layer. Aside from parallel fibers, the molecular layer also contains the dendrites of the underlying Purkinje neurons as well as

scattered two types of inhibitory interneurons, stellate and basket cells, which are responsible for balancing the excitation on the Purkinje cells.

1.2.2.2 Inhibitory Interneurons

Basket cells are only found in mammals and birds (Llinás and American Medical Association Education and Research Foundation. Institute for Biomedical Research., 1969), with a ratio of one per six Purkinje cells. One basket cell functions as an inhibitory interneuron to about eight to nine Purkinje cells. Its dendritic tree expanses to the pial surface and make synapses with the parallel fibers as well. The axon of basket cell runs above the row of Purkinje cells and perpendicularly to the axis of the folium. Stellate cells, the other inhibitory interneuron in the molecular layer, are located in the outer two-thirds of the molecular layer. Their dendrites form inhibitory synapses with the Purkinje cell dendrites. In addition, basket cells, stellate cells, and Purkinje cells all share the same parallel fiber input. Aside from basket and stellate cells, as mentioned earlier, Lugaro and Golgi cells are inhibitory interneurons in the granular layer. Lugaro cells locate just under the Purkinje cells. Their dendrites receive massive innervation from Purkinje cell axon collaterals and their axons reach out into the molecular layer where Lugaro cells form plenty of symmetrical gamma-aminobutyric acid-(GABA)ergic synapses with basket and stellate cells (Laine and Axelrad, 1998). Golgi cells, more interestingly, are negatively regulated by Purkinje cell collaterals but inhibit the synaptic transmission between mossy fiber rosettes and granule cell dendrites. Because the dendritic arbor of Golgi cells also receives excitatory synaptic input from the parallel fibers, from the

connections, the gate of mossy fiber input is tightly controlled by Purkinje cells.

1.2.2.3 Purkinje cells

Underneath the molecular layer is the Purkinje cell layer, which is composed of a single layer of Purkinje neurons, separating the outer molecular layer from the inner granular layer. The Purkinje neurons have large cell bodies (50~80 μm) and their extensive dendritic trees perpendicular to the axis of a folium reach out into the molecular layer. All Purkinje neurons are characterized as inhibitory GABAergic (Ito and Yoshida, 1964) projection neurons and they are the sole output of the cerebellar cortex. One given Purkinje cell may form as many as 100,000 synapses with other neurons, which is more than any other neuron studied. Their axons leave the lower rounded pole, widen, and become myelinated. In the granular layer, these axons discharge many collaterals oriented perpendicularly to the folium axis. They further extend down into the underlying white matter where they target the deep nuclei and vestibular nuclei. A typical Purkinje cell gives off 500 terminals to contact 35 cerebellar nuclei.

1.2.2.4 Deep cerebellar nuclei

The cerebellar nuclei are the target of the Purkinje cell axons, with an average ratio of one per 26 Purkinje cells. A nuclear cell receives a dense Purkinje cell innervation, of which about 14 inhibitory terminals are from the same Purkinje cell and 12,000 terminals from 860 different Purkinje cells. It has been shown that glutamate is the neuromediator (Schwarz and Schmitz, 1997) and the excitatory input for the cerebellar nuclei from collaterals of the

climbing and mossy fibers. However, the mossy fibers are the major excitatory input to the nuclear cells. Interestingly, in some cases, the cerebellar nuclei project to vermis where the Purkinje cells do not make projections back to the dentate nucleus. It suggests that these connections may be presented either as a negative feedback or as an open communication between different areas of cerebellum.

1.2.2.5 Mossy fibers and climbing fibers

Mossy fibers are characterized as the major glutamatergic and excitatory inputs to the cerebellum. The sources of mossy fibers include the cortex, the vestibular nerves and nuclei, the spinal cord (Matsushita et al., 1979), the reticular formation, and feedback from cerebellar nuclei (Gould, 1979; Tolbert et al., 1978). Different axons ascend into the white matter of the cerebellum where they contact with deep cerebellar nuclei as well as innervate granule cells at the cerebellar glomeruli. Therefore, the mossy fibers are able to pass the sensory information to the granule cells, which then send it along the parallel fibers to the Purkinje cells for processing.

The other excitatory input to the cerebellum is climbing fibers. The sources of climbing fibers are axons from the inferior olivary nucleus located in the medulla (Desclin, 1974; Sotelo et al., 1975). The axons enter the cerebellum and make synapses with the deep cerebellar nuclei and Purkinje cells. A recent study has shown that climbing fibers play an important role in motor behaviors (McKay et al., 2007).

1.3 Purkinje Cell

1.3.1 Purkinje cell development

Purkinje cell neuroblasts of the cerebellum arise from the anterior metencephalon. Between E10 and E13, the Purkinje cell precursors undergo their final mitosis in the subventricular germinal zone (Miale and Sidman, 1961) and then commit to become Purkinje cells (Leclerc et al., 1988; Oberdick et al., 1993; Seil et al., 1995; Wassef et al., 1990). The post-mitotic Purkinje cells migrate into the anlage of the cerebellum via radial glial guidance (Edwards et al., 1990). Initially, they aggregate together and form a layer with 10–15 cells thick. The undeveloped Purkinje cell layer further divides into clusters (Armstrong and Hawkes, 2000; Hawkes and Eisenman, 1997; Herrup and Kuemerle, 1997). After E14, these Purkinje cell clusters make contacts with newly entered mossy and climbing fibers (Arsenio Nunes and Sotelo, 1985; Chedotal et al., 1997; Grishkat and Eisenman, 1995; Ji and Hawkes, 1995). Consequently, clusters of Purkinje cells scatter to form a monolayer at around birth. Studies have shown that the Reelin-Dab1 pathway (Gallagher et al., 1998; Howell et al., 1997; Sheldon et al., 1997), cadherins (Gilmore and Herrup, 2000), and integrins (Dulabon et al., 2000) are involved in the scattering process of Purkinje cells. Finally, most Purkinje cells express calbindin uniformly in the postnatal period and develop elaborate dendritic arbors as well as synapses with granule cells during the first four postnatal weeks.

1.3.2 Activity regulation of Purkinje cells

The Purkinje cells are the exclusive output of the cerebellar cortex and their activity is delicately regulated by two distinct excitatory fiber systems and three different local inhibitory interneurons in the cerebellum. Both fiber

systems, mossy fibers and climbing fibers, carry information flowing from the cerebral cortex or medulla into the cerebellum and initially influence on the deep cerebellar nuclei. Their collateral axon branches make contacts with the deep cerebellar nuclei, which form the primary cerebellar circuit. Interestingly, mossy fibers and climbing fibers inputs are each regulated distinguishably in response to sensory stimulation and motor actions. Mossy fibers excite Purkinje cells indirectly through local clusters of granule cells in cerebellar glomeruli. First, the mossy fibers activate the granule cells and then the granule cell axons, the parallel fibers, make robust excitatory connections with nearby Purkinje cells. Each Purkinje cell receives input from nearly 200,000 parallel fibers, which collect excitatory input from many mossy fibers. Similar to the mossy fibers, the synapses of climbing fibers are all excitatory. The climbing fibers can transiently enhance the influence of mossy fiber inputs on Purkinje cells as well as induce large excitatory postsynaptic potentials in both the soma and dendrites of the Purkinje cells. Despite the primary circuit initially activating Purkinje cells, the initial excitation is further modulated by the inhibitory action of the Purkinje neurons.

The activity of the Purkinje cells is also regulated by local inhibitory interneurons- basket, stellate, and Golgi cells - which all receive excitatory inputs from the parallel fibers but function in different ways. The axons of basket cells make contact with the cell bodies of more distant Purkinje cells. As a result, the excited basket cells inhibit the Purkinje cells outside where the excitation occurs. In contrast to basket cells, suppression from stellate cells is via their short axons contacting nearby dendrites of Purkinje cells. More indirectly, Golgi cells inhibit Purkinje cells through the parallel fibers by

reducing the duration of excitation on granule cells within the cerebellar glomeruli.

1.3.3 Death of Purkinje cell

Dysfunctional Purkinje cells in mice are often linked to several characteristic clinical symptoms, such as abnormal postures, gait abnormalities, tremor, and ataxia. Death of Purkinje cells is also exhibited in an atrophic cerebellum. If the Purkinje cell death occurs during the neonatal stage, the loss of cell bodies in Purkinje cell clusters results in the Purkinje cell layer containing fewer gaps in the adult cerebellum. In addition, the early death of Purkinje cells in development typically causes a reduced volume of cerebellum and severe morphological abnormalities (Millonig et al., 2000). In contrast, when the death of Purkinje cells happens in the adult cerebellum, it presents gaps both in the Purkinje cell layer and the molecular layer.

Detection of whether the Purkinje cell undergoes naturally-occurring cell death during development remains unclear. There is no Purkinje cell death from P4 to adulthood (Caddy and Biscoe, 1979). Double positive staining of CaBP and TUNEL or active caspase-3 antibody of Purkinje cells in P3-P4 cerebellum (Kitao et al., 2004; Marin-Teva et al., 2004) suggests that Purkinje cells engage in programmed cell death at P3-P4. However, it has been shown that caspase-3 mRNA is expressed at high levels in the Purkinje cell layer from P1 to P8 (de Bilbao et al., 1999). Active caspase-3 is present at all stages of Purkinje cell dendritic remodeling during the first 6 postnatal days (Madalosso et al., 2005). In addition, the developing Purkinje cells intensely express the pro-survival factor *bcl-x* mRNA in the developing cerebellum (Frankowski et al., 1995). The evidence obtained from transgenic mice has

shown that over-expressing Bcl-2 or inactivating Bax increases the number of Purkinje cells in young adult mice in the cerebella (27–40% for *Bcl-2* transgenic mice (Zanjani et al., 1996) and approximately 30% for the *Bax*$^{-/-}$ (Fan et al., 2001). However, in older *Bcl-2* transgenic mice, the numbers of Purkinje cells in the cerebella are reduced at 6-months of age and then ultimately reach the control level by 18-months of age. The reduction in cell number correlates with the expression pattern of the exogenous human Bcl-2. It indicates that sustained expression of Bcl-2 is responsible for Purkinje cells survival (Zanjani et al., 2004).

A study has shown that autophagy function as an anti-apoptotic clearance mechanism by discarding altered mitochondria (Brunk and Terman, 2002). In addition, deficiency of autophagy leads to loss of the Purkinje cells (Hara et al., 2006; Komatsu et al., 2006). Autophagy, however, has been also proposed to be involved in neuronal cell death (Chang et al., 2003; Lang-Rollin et al., 2003; Xue et al., 1999). Inactivation of some *atg* components arrests neuronal cell death (Yuan et al., 2003). Therefore, whether the autophagy is responsible for the neuronal cell death remains controversial.

It has been widely documented that the death of Purkinje cells often occurs along degenerative changes during aging. In older animals, the numbers of Purkinje cells have been shown to be decreased (Amenta et al., 1994; Sturrock, 1989) and as many as 14% of Purkinje cells exhibit axonal spheroids or torpedoes (Baurle and Grusser-Cornehls, 1994). Moreover, degenerative Purkinje cells show the decreased activity of succinic dehydrogenase within mitochondria (Fattoretti et al., 1998), implying that degenerative changes during aging may be related to oxidative stress (Bickford et al., 2000). Besides, studies have shown that there are multiple

defects contributing to Purkinje cell loss as a secondary effect. These defects include the neurodegeneration of inferior olivary neurons (Ghetti et al., 1987; Shojaeian et al., 1988; Triarhou and Ghetti, 1991) or deep cerebellar nuclei (Triarhou et al., 1987; Wassef et al., 1986), and a significant loss of granule cells.

1.4 Protein quality control systems and neurodegeneration

Proteins require proper folding to be fully functional after they are synthesized. To secure the integrity of cellular functions, the protein quality control systems play an essential role among various regulation machineries in a cell. Misfolded proteins are often non-functional and, sometimes, they form aggregates which may be toxic to the cells. Because protein misfolding can happen at different locations and anytime in the cell, protein quality control systems have evolved into complex mechanisms, including recognition of aberrant proteins, deposition of misfolded proteins in inclusion bodies, and degradation of targeting proteins, to protect cells from accumulation of misfolded proteins. However, the protein quality control systems may be impaired owed to aging or mutations. The deficiency in protein quality control systems could further increase the presence of end-stage protein aggregates. Although it is still under debate whether the accumulation of protein inclusions is neurotoxicity or neuroprotection, most neurodegenerative diseases have been characterized by deposits of protein aggregates. Neurodegenerative disorders often produce and accumulate abnormal proteins within cells. The progressive intracellular accumulation of aberrant proteins may be resulting from the imbalance between the capability or activity of these quality control systems and the misfolded proteins. The

protein aggregates may further induce damages on cellular functions and eventually lead to cell death.

1.4.1 Ubiquitin-proteasome system (UPS)

The ubiquitin-proteasome system (UPS) is known as the primary cellular protein degradation system (Figure 1.4). Most proteins tagged with polyubiquitin chains are destined for degradation by the proteasome. The ubiquitination involves a cascade of enzymes (Gao and Karin, 2005). The activation of ubiquitin by the enzyme E1 is the initial step of the process. The enzyme E1 forms a thiol-ester bond between its cysteine and the final glycine of ubiquitin and then transfers the ubiquitin to enzyme E2 via a trans-thio-esterification reaction. The final step of ubiquitination requires the activity of a specific enzyme E3 which creates an isopeptide bond between the C-terminal glycine of ubiquitin and a lysine of the target protein. Interestingly, ubiquitination has a hierarchical organization. There are about ten E1s, about hundreds E2s and possibly thousands of E3s in humans (Hicke et al., 2005). More important, different E2/E3 combinations lead to the linkage-specific ubiquitin chains, such as K48 and K63 linkages. Of which, K48 linkages are the main peptide signal for degradation by the proteasome. Studies have revealed several proteins, such as RAD23, DSK2 and CDC48, that are responsible for transfering the polyubiqutinated proteins to the proteasome (Elsasser and Finley, 2005; Richly et al., 2005). The proteasome consists of a 20S catalytic core and a 19S lid. In the 19S lid, the S5a subunit initiates proteolysis by binding to polyubiquitinated proteins. The polyubiquitin chains are further cleaved by deubiquitinating enzymes. In addition, the target protein is unfolded and degraded by the three enzymatic activities: trypsin-like, chymotrypsin-like,

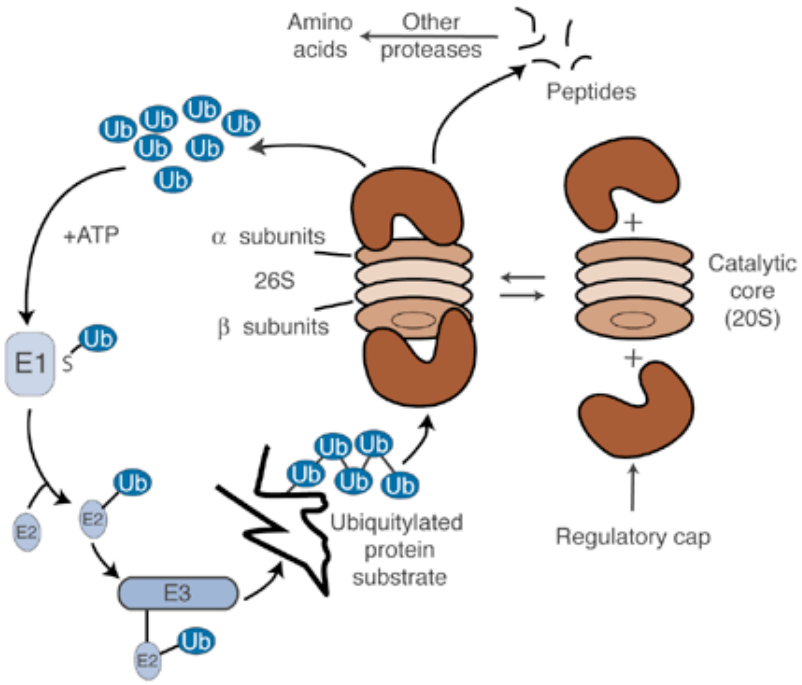


Figure 1.4 | Schematic representation of the ubiquitin–proteasome system. The proteasome consists of a catalytic core and two regulatory caps. The core resembles a barrel–like structure consisting of four stacked rings that are made up of two types of subunits. One of the subunits is the site of the proteinase activities responsible for protein degradation. The two caps recognize and bind to proteins that are destined for degradation and help these proteins enter the channel in the center of the catalytic core. There, the proteins are cut into peptides that then can be degraded further into amino acids. Proteins to be degraded by the proteasome first must be marked by the addition of at least four ubiquitin molecules (Ub). Transfer of ubiquitin to the target proteins is mediated by ubiquitin–activating enzymes (E1), conjugating enzymes (E2), and ligating enzymes (E3). This process requires energy, which is provided by the form of adenosine triphosphate (ATP), the cell's primary energy source. †From (Donohue, 2002), courtesy of the author.

and PGPH-like in the 20S catalytic core of proteasome (Pickart and Cohen, 2004). Studies have shown that the UPS system plays an essential role in regulation of the cell cycle (Hershko, 2005) and transcription (Auld and Silver, 2006; Muratani and Tansey, 2003), in modification of synaptic plasticity (Yi and Ehlers, 2005), and in cellular protein quality control (de Vrij et al., 2004; Goldberg, 2003; Kopito, 2000; Scheper and Hol, 2005; Varshavsky, 2005).

1.4.2 Autophagy-lysosome system

Studies have also shown that the UPS only degraded the soluble form (Betarbet et al., 2005) but not fibrillar forms of the aggregated proteins (Stefanis et al., 2001). In order to remove the fibrillar protein aggregates, cells adopt another protein quality control system, the autophagy-lysosome system. Unlike the ubiquitin-proteasome system mainly for the degradation of short-lived proteins, the autophagy-lysosome system is responsible for the degradation of long-lived proteins, organelles turn-over, and the cellular survival response to starvation (Reggiori and Klionsky, 2005). Generally, the initial step of autophagy is the formation of the autophagosome, which is a double membrane structure containing several autophage-related proteins (Atgs). The conjugation of Atg12 and Atg5 is responsible for the double membrane elongation (Reggiori and Klionsky, 2005). Subsequently, the outer membrane fuses with a lysosome and the cargos within the autophagosome are released to be degraded (Levine and Yuan, 2005). Additionally, it has been shown that the deficiency of autophagy-lysosome system induces aggresomes to inclusion bodies (Kopito, 2000).

Autophagy can be classified as two types, selective and non-selective autophagy, based on the different sources of stimulation. Selective autophagy

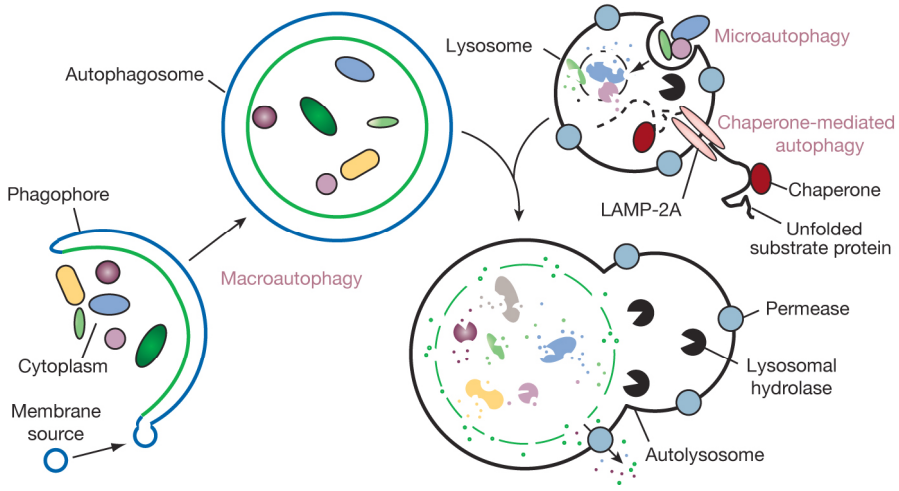
is initiated by intracellular components; non-selective autophagy is triggered by extracellular stimuli, such as starvation (Reggiori and Klionsky, 2005). In addition, autophagy can be cataloged as macroautophagy, microautophagy, and chaperone-mediated macroautophagy (CMA) (Larsen and Sulzer, 2002) (Figure 1.5). Under cell stress or starvation, macroautophagy is initiated to degrade bulk proteins, and chaperone-mediated macroautophagy is triggered to specifically remove proteins with a KFERQ motif. Microautophagy functions under basal conditions to eliminate cytoplasmic proteins. A recent study has shown that autophagy can be triggered by the impairment of the UPS to degrade aggresomes in mammalian cells as well (Iwata et al., 2005a; Iwata et al., 2005b). Interestingly, activation of macroautophagy is mTOR activity-dependent and it has been suggested that macroautophagy functions as a compensatory response to the malfunction of the UPS in neurons (Rideout et al., 2004) (Figure 1.6). Studies have demonstrated that following the inhibition of proteasome activity neuronal autophagy is initiated in cultured neuroblastoma cells (Ding et al., 2003), suggesting that cells protect themselves from stress by activation of the lysosomal system (Larsen and Sulzer, 2002).

1.4.3 Neurodegenerative diseases

Studies have reported that many chronic neurodegenerative diseases correlate with accumulation of ubiquitin-conjugated protein inclusion bodies, such as Alzheimer's disease (AD) with neurofibrillary tangles, Parkinson's disease (PD) with brainstem Lewy bodies (LBs), Amyotrophic Lateral Sclerosis (ALS) with Bunina bodies, Huntington's disease with polyglutamine extension (Poly-Q) in nuclear inclusions, Spinocerebellar Ataxias (SCAs), and Spinal and Bulbar Muscular Atrophy (SBMA; Kennedy's disease)

Figure 1.5 | Different types of autophagy.

Microautophagy refers to the sequestration of cytosolic components directly by lysosomes through invaginations in their limiting membrane. The function of this process in higher eukaryotes is not known, whereas microautophagy-like processes in fungi are involved in selective organelle degradation. In the case of macroautophagy, the cargoes are sequestered within a unique doublemembrane cytosolic vesicle, an autophagosome. Sequestration can be either nonspecific, involving the engulfment of bulk cytoplasm, or selective, targeting specific cargoes such as organelles or invasive microbes. The autophagosome is formed by expansion of the phagophore, but the origin of the membrane is unknown. Fusion of the autophagosome with an endosome (not shown) or a lysosome provides hydrolases. Lysis of the autophagosome inner membrane and breakdown of the contents occurs in the autolysosome, and the resulting macromolecules are released back into the cytosol through membrane permeases. CMA involves direct translocation of unfolded substrate proteins across the lysosome membrane through the action of acytosolic and lysosomal chaperone hsc70, and the integral membranereceptor LAMP-2A (lysosome-associated membrane protein type 2A). †From (Mizushima et al., 2008), courtesy of the author.


Autophagosome
Phagophore
Cytoplasm
Membrane
source
Macroautophagy
Lysosome
Microautophagy
Chaperone-mediated
autophagy
LAMP-2A
Chaperone
Unfolded
substrate protein
Permease
Lysosomal
hydrolase
Autolysosome

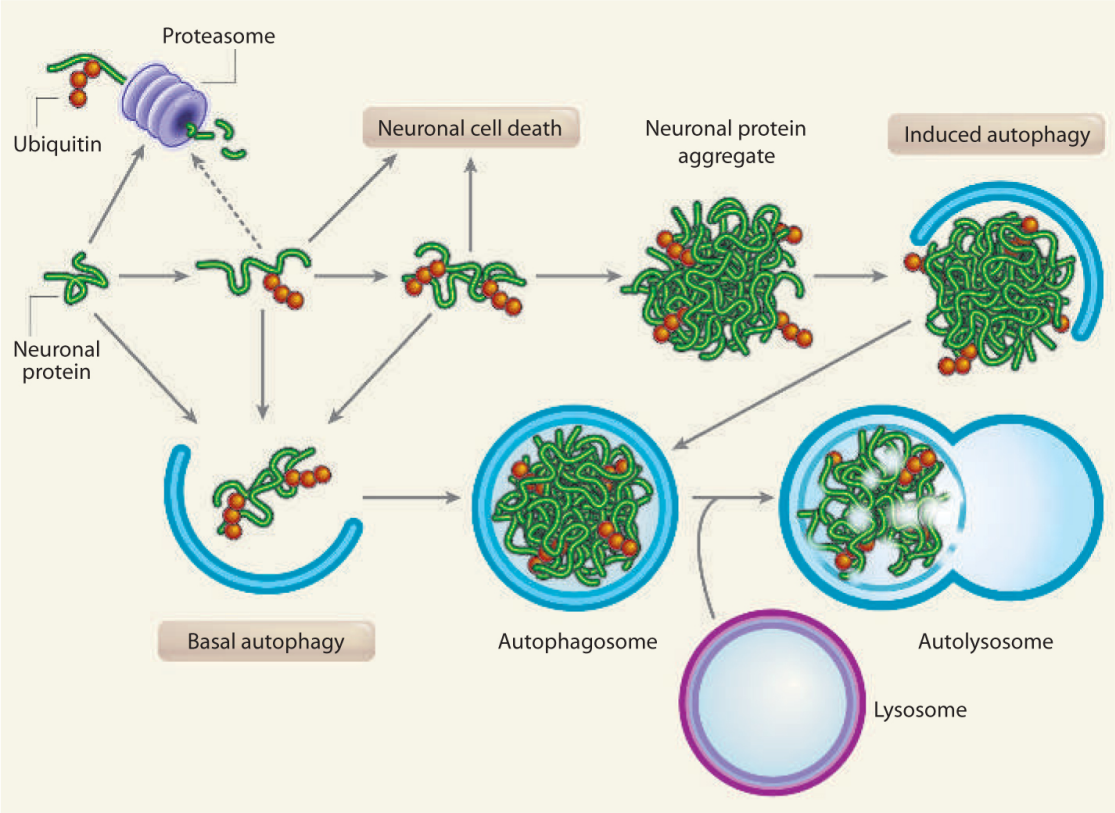


Figure 1.6 | The role of autophagy in protecting against neuronal cell death. Certain key neuronal proteins may misfold. These misfolded proteins can become ubiquitinated (red circles) and degraded by the proteasome. However, these proteins may be poor substrates for the proteasome, and instead will accumulate in the cytoplasm, form aggregates and possibly leading to cell death. Basal autophagy can keep the levels of these proteins low enough to prevent toxic effects by sequestering them inside autophagosomes that deliver them to the lysosome for subsequent degradation. The misfolded proteins may also form large aggregates or inclusion bodies that can induce an autophagic response. These large aggregates may be more protective than harmful. †From (Klionsky, 2006), couresy of the author.

(Alves-Rodrigues et al., 1998; Sherman and Goldberg, 2001). However, the pathological significance of the protein inclusions remains unclear. Some studies in PD showed that accumulation of ubiquitin conjugates in LBs could be a secondary effect resulting from the impairment of the UPS. The accumulation process may involve several cellular machineries, including formation of aggresomes (Johnston et al., 1998), and relocalization of proteasome to aggregates, to clear the abnormal protein inclusions (Fabunmi et al., 2000). In addition, recent studies indicate that the UPS can be inhibited by soluble aggregated protein inclusions (Bence et al., 2001). Conversely, some studies suggest that the concentration of ubiquitin conjugates in inclusion bodies is a protective mechanism and may separate the aggregates from the vulnerable cellular machineries.

In summary, the affected regions with the presence of protein inclusions and the content of the protein aggregates are disease-specific in neurodegenerative disorders. The cellular mechanisms behind the pathology remain poorly understood.

1.4.3.1 Alzheimer's Disease (AD)

In 1906, Alois Alzheimer first delineated the neuropathological changes in AD involving abnormal protein aggregation with gradual neuronal loss and progressive dementia. The protein aggregates in AD are characterized as extracellular amyloid plaques and intracellular neurofibrillary tangles (Hardy and Selkoe, 2002). The extracellular aggregates are rich in amyloid β peptides (Aβ) resulting from proteolytic cleavage of the cellular surface protein amyloid precursor peptide (APP) by β and γ secretase functioning at specific sites. Hence, the cleaved fragments (Aβ1−40 or Aβ1−42) misfold and form the

extracellular amyloid plaques. The intracellular aggregates of the neurofibrillary tangles are rich in Tau protein. Tau protein undergoes increased hyperphosphorylation during the formation of neurofibrillary tangles. Interestingly, mutant APP but not mutant tau is sufficient to cause AD, indicating that accumulation of Aβ is the central event of AD pathogenesis.

Alzheimer's disease and the UPS

Aβ is normally degraded via the UPS in cultured neurons and astrocytes and inhibition of the UPS increases Aβ-induced cell death (Lopez Salon et al., 2003). Studies have shown that AD patients exhibit the impairment of proteasome activity in the brain (Keller et al., 2000) as well as a reduced activity of E1 and E2 enzymes in cerebral cortex (Lopez Salon et al., 2000). However, some studies show that amyloid has no effect on proteasome activity (Lue et al., 1999); other reports show that no alteration of proteasome activity is found in AD patients (Blandini et al., 2006). In addition, it remains unknown whether the impairment of the UPS activity cause Aβ plaques, or Aβ/Tau leads to proteasome inactivation.

Alzheimer's disease and Autophagy

Before the formation of amyloid plaque, upregulated endocytic-lysosomal and autophagy-lysosomal system is also found in AD (Cataldo et al., 1995; Nixon et al., 2005) to successfully remove the aggregated proteins (Nixon et al., 2005). However, as the disease progresses, the decreased efficiency of lysosomal system (Yu et al., 2005) and the failure of fusion of autophagosome to lysosomes leads to numerous autophagosomes accumulated in neurons (Nixon et al., 2005). A recent report has proposed that

the autophagosomes may eventually become the internal source for Aβ deposition because they contain the APP on the membrane and secretases (Yu et al., 2005).

1.4.3.2 Amyotrophic Lateral Sclerosis (ALS)

The neuropathology of ALS has been well characterized with primarily neuronal death of brainstem and spinal motorneurons, corticospinal degeneration, and paralysis of skeletal muscle. The mutations in all ALS are involved in the gene coding for Copper-Zinc superoxide dismutase (SOD1) (Valentine and Hart, 2003), which scavenges oxygen radicals to prevent cells from oxidative stress.

Amyotrophic lateral sclerosis and the UPS

The mutant SOD1 proteins tend to be more insoluble (Johnston et al., 2000; Shinder et al., 2001) and form polyubiquitinated aggregates in Bunina bodies, consisting of proteasome and neurofilaments (Alves-Rodrigues et al., 1998; Johnston et al., 2000). Unlike other neurodegenerative protein inclusions, the mutant SOD1 aggregates do not cause cell death (Lee et al., 2002) and can be further degraded by the UPS (Hoffman et al., 1996; Johnston et al., 2000). Although its role remains unclear, the accumulation of mutant SOD1 aggregates is enhanced by impairment of defense against oxidative stress and inhibition of the UPS (Lee et al., 2002). A recent study has shown that the expression of mutant SOD1 induces oxidative stress to cells (Hyun et al., 2003). In addition, over-expression of the E3 ligase dorfin can facilitate the removal of mutant SOD1 via the UPS, leading to increased cell survival (Niwa et al., 2002). In ALS patients' spinal cord, it is often found that an increase of

dorfin expression (Ishigaki et al., 2002). It has been proposed that under increasing oxidative stress, the cell accumulates the mutant SOD1 protein inclusions more quickly and the accumulating mutant SOD1 protein inclusions can eventually inhibit the UPS (Urushitani et al., 2002). Consequently, the inhibition of the UPS increases the mutant SOD1 protein inclusions to form aggresomes (Johnston et al., 2000).

1.4.3.3 Parkinson's Disease (PD)

The neuropathological character of PD is the progressive and extensive loss of dopaminergic neurons in the area of substantia nigra pars compacta and the stratium. Many dopaminergic neurons also present intracellular aggregated proteins, such as α-synuclein, in Lewy bodies (Nussbaum and Polymeropoulos, 1997; Pollanen et al., 1993). It has been proposed that mitochondrial dysfunction mediated oxidative stress and impairment in the major proteolytic systems both contribute to the accumulation of protein aggregates, which is responsible for loss of dopaminergic neurons (Abou-Sleiman et al., 2006; Betarbet et al., 2005). Studies have shown that mutations in DJ-1 (Mitsumoto and Nakagawa, 2001) or PINK1 (Valente et al., 2004) lead to mitochondrial dysfunction, causing increased oxidative stress. Besides, mutations in Parkin (Kitada et al., 1998), UCH-L1 (McNaught et al., 2002b), components of the ubiquitin-proteasome system, and ATP13A2 (lysosomal ATPase) (Ramirez et al., 2006) can result in deficiency in protein quality control systems. Interestingly, recent studies have revealed that a genetic interaction between Parkin and PINK (Pallanck and Greenamyre, 2006; Park et al., 2006). In summary, the death for dopaminergic neurons may result from

loss-of-function of several different proteins because of their aggregation or the toxic gain-of-function of the aggregated protein inclusions.

Parkinson's Disease and the UPS

In PD, the UPS is highly involved in the pathogenesis of neurodegeneration. Parkin (PARK2) is one of the important molecules involved in the pathogenesis of PD. It has been identified as an ubiquitin-protein ligase functioning with UbcH7 and UbcH8 (Imai et al., 2000; Shimura et al., 2001). Parkin can recruit E2 ligase of the ubiquitination machinery as well as associate with RPN10 (S5a) subunit of the 26S proteasome (Sakata et al., 2003; Upadhya and Hegde, 2003). Therefore, Parkin transfers the polyubiquitnated substrates to the degradation machinery. Additionally, inactivation of Parkin or deficiency in interaction of Parkin with its partners has been proposed to play key role in PD pathogenesis (Dawson and Dawson, 2003; Dev et al., 2003; McNaught and Jenner, 2001; Mizuno et al., 2001). Hence, mutant Parkin could cause accumulation of abnormal proteins, resulting in death of dopaminergic neurons. An *in vivo* study has shown that Drosophila with Parkin knockout presents reduced life span, locomotor disorders, and selective male sterility (Greene et al., 2003). Of which, the locomotor disorder is caused by mitochondrial dysfunction in muscle cells. It is known that mitochondrial dysfunction often leads to cell death.

One of the most common familial forms of PD is AR-JP (autosomal-recessive Parkinson's disease [ARPD]) (Kitada et al., 1998). Various deletion and point mutations of Parkin lead to its inactivation (Lucking et al., 2000) or loss of the ability to interact with partners, such as CHIP (carboxy-terminus of the sc70-Interacting protein) (Imai et al., 2002). A recent study has suggested

that insufficient activity of Parkin causes some cases of AR-JP (West et al., 2002). In addition, studies have characterized several Parkin substrates linked to AR-JP, including cell-division control related protein (CDCrel-1) (Zhang et al., 2000), Parkin-associated endothelial- like (Pael) receptor (Imai et al., 2001), 22 kDa form of O-glycosylated α-Synuclein (αSp22) (Shimura et al., 2001), Synphilin-1 (Chung et al., 2001), polyglutamine Ataxin-3 fragment (Tsai et al., 2003), and cyclin E (Staropoli et al., 2003). Although these substrates are regulated by Parkin-mediated polyubiquitination, it is still unclear whether Parkin-inactivation-induced accumulation of these proteins play a role in cytotoxicity to dopaminergic neurons.

As mentioned above, αSYN (also termed PARK1) is one of the Parkin substrates and identified as another important molecule causing PD via its mutant affecting the UPS activity. In PD patients, αSYN is often found at high intracellular concentration which leads to protein oligomerization (Singleton et al., 2003). The oligomerization of αSYN further results in progressive aggregation of αSYN fibrils in Lewy bodies (Conway et al., 2000). Hence, the αSYN protein aggregates increase the sensitivity of neuronal cell to toxic agents as well as inhibit proteasome activity, leading to cell apoptosis (Lee et al., 2001). Moreover, it has been shown that normal and aggregated αSYN can both interact with S6′ subunit of proteasome to further inhibit proteasomal function (Bence et al., 2001; Snyder et al., 2003). Malfunctional αSYN may also affect its associated proteins, such as Synphilin (Chung et al., 2001) and tyrosine hydroxylase (Doskeland and Flatmark, 2002) to disturb neuronal homeostasis and develop neurodegeneration.

There is increasing evidence suggesting deficient the UPS in PD. Studies have shown that loss of α subunits of the 26S proteasome (McNaught et al.,

2002a) impairs the proteasomal catalytic activity in sporadic PD (McNaught and Jenner, 2001). In addition, decreased 19S/PA700 proteasome activator as well as the PA28 regulator are found in the brain of PD patients (McNaught et al., 2003).

Parkinson's Disease and Autophagy

Aside from the UPS, αSYN can be degraded by CMA as well (Webb et al., 2003). However, αSYN mutants bind to the CMA with high affinity, resulting in blockage of cargo transfer and substrate degradation (Cuervo, 2004). Hence, normal substrate proteins in CMA cannot be turned over and accumulate in cells (Cuervo, 2006). Interestingly, inhibition of the ubiquitin-proteasome system and CMA can increase activity of macroautophagy (Iwata et al., 2005b; Massey et al., 2006) to eliminate both the cytosolic toxic and aggregated αSYN (Rideout et al., 2004).

1.4.3.4 Polyglutamine diseases

The pathogenetic character of polyglutamine diseases is a mutation with the CAG triplet repeat expansion (Gusella and MacDonald, 2000). Although various combinations of motor, psychiatric, cognitive, and sensory symptoms may exhibit in the different polyglutamine expansion (Nakamura et al., 2001; Ross, 2002; Taylor et al., 2002; Zoghbi and Orr, 2000), most of the polyglutamine diseases are autosomal-dominant and have specific disease-related misfolded proteins in inclusions. These diseases are considered as a gain in toxicity from accumulating cytoplasmic or intranuclear protein inclusions (Ross, 1997; Scherzinger et al., 1997; Zoghbi and Orr, 2000). The process of polyglutamine-containing protein accumulation involves multiple

steps, including a nucleation event (Perutz and Windle, 2001), action of transglutaminase (Kahlem et al., 1996), transfer of polyglutamine-containing protein via the microtubule organization center (MTOC or centrosomes) to a perinuclear localization (Hoffner et al., 2002; Waelter et al., 2001).

Among these polyglutamine diseases, Huntington's disease (HD) is the best studied disorder (Landles and Bates, 2004). The neuropathic features of HD are death of neuronal cells, progressive cognitive impairment, abnormality of movement, and intraneuronal inclusion bodies of mutant Huntingtin (Kegel et al., 2000). Mutant Huntingtin is because of genetic polyglutamine expansion in the N-terminal sequence of Huntingtin (Zuccato et al., 2003). The mutant Huntingtin forms aggregates and promotes aggregation of the wild-type Huntingtin (Ambrose et al., 1994; Busch et al., 2003). Because Huntingtin involved in different intracellular processes, such as gene transcription and intracellular trafficking (Li and Li, 2004) via interacting with various proteins at its N-terminal region, the aggregation of Huntingtin with other proteins increase the effect of loss-of-function (Cha, 2000; Gidalevitz et al., 2006).

Polyglutamine diseases and the UPS

Studies have indicated that several elements of the UPS are co-aggregated with different disease-specific polyglutamine proteins (Chai et al., 1999; Cummings et al., 1998; Schmidt et al., 2002; Stenoien et al., 1999; Stenoien et al., 2002; Verhoef et al., 2002; Waelter et al., 2001). For instance, ubiquitinated Huntingtin interacts with the E2 enzyme (Kalchman et al., 1996), Ataxin-7 associates with the S4 subunit of the proteasome (Matilla et al., 2001), and Ataxin-3 binds to the shuttle protein Rad23 and the S5a subunit (Doss-Pepe et

al., 2003). From an *in vivo* report, transgenic Ube3a null mice which express mutant Ataxin-1 exhibit less intranuclear aggregates within neurons (Cummings et al., 1999) owed to the deficiency of polyubiquitination on Ataxin-1. In addition, *in vitro* studies show that over-expression of mutant Huntingtin causes inhibition of proteasome activity (Bence et al., 2001; Ding et al., 2002) and expression of truncated Huntingtin aggregates more rapidly when the proteasome activity is pharmacologically inhibited (Lunkes et al., 2002). Furthermore, there are several studies showing that reducing transglutaminase activity in the brain can prolong life span of the R6/2 transgenic mouse model of HD (Dedeoglu et al., 2002; Karpuj et al., 2002), suggesting that polyglutamine-expansion proteins may be stabilized by the reaction of transglutaminase. Therefore, the transglutaminase-modified polyglutamine-containing proteins block the proteasome and they are free from degradation by the UPS system. More interestingly, a study using cultured mouse neuroblastoma cells reported that mutant Huntingtin inhibits the proteasome catalytic activity, p53 degradation, and normal mitochondrial membrane potential, leading to release of cytochrome c, activation of caspase-cascade, and apoptosis (Jana et al., 2001).

Polyglutamine diseases and Autophagy

Studies have shown that striatal neurons derived from R6/2 transgenic HD mice exhibit increased autophagy (Petersen et al., 2001). In addition, deficiency in macroautophagy is found to associate with Huntington's disease (Qin et al., 2003; Ravikumar et al., 2002) and the formation of Huntingtin aggregates (Qin et al., 2003; Ravikumar et al., 2002; Shibata et al., 2006). Furthermore, activation of macroautophagy facilitates the removal of

Huntingtin, improving the neurological symptoms in mouse models of Huntington's disease (Ravikumar et al., 2004).

1.4.3.5 Prion Disease

In 1982, Stanley Prusiner originally proposed a proteinaceous infectious hypothesis to describe the underlying mechanism of Prion diseases. Owed to the unique conformational change of the brain, the Prion disease is also named as transmissible spongiform encephalopathies (TSEs). TSEs includes bovine spongiform encephalopathy (BSE) in cattle (Lampert et al., 1972), Scrapie in sheep and goats (Lampert et al., 1972), chronic wasting disease (CWD) in elk and deer (Johnson, 2005; Lampert et al., 1972), as well as Creutzfeld-Jakob disease (CJD) (Ironside et al., 1993; Lampert et al., 1972; Masters and Richardson, 1978; Piccardo et al., 1990; Voigtlander et al., 2001), fatal familial insomnia (FFI) (Glatzel et al., 2005; Johnson, 2005; Lampert et al., 1972), Gerstmann-Sträussler-Scheinker disease (GSS) (Glatzel et al., 2005; Johnson, 2005; Lampert et al., 1972), and kuru in human (Lampert et al., 1972; Piccardo et al., 1990). The neuropathology of Prion disease is characterized by extensive neuronal cell death, spongiform degeneration, reactive gliosis, and extracellular amyloid aggregates. Therefore, Prison disease progressively develops motor disturbance and dementia causing death.

The normal prion protein (PrP) is called PrP^C, which is particularly abundant in the immune and nervous systems and functions as a copper binding protein with antioxidative activity (Brown, 2002). PrP^C associates with chaperone BiP during protein folding (Jin et al., 2000) and normally undergoes degradation by the UPS via endoplasmic reticulum associated protein degradation (ERAD) (Yedidia et al., 2001; Zanusso et al., 1999). In TSEs, PrP^C is

misfolded to its insoluble format, PrP-scrapie (PrP^{Sc}). The conformational change from PrP^{C} to PrP^{Sc} can be further induced by PrP^{Sc}. Consequently, the accumulating PrP^{Sc} isoform causes cell death, resulting in release of PrP^{Sc} which can affect the neighboring cells.

Polyubiquitin protein inclusions have been found in CJD brains (Ironside et al., 1993), suggesting an overload of the UPS or a decrease of proteasome activity. Additionally, it has been suggested that malfunction of the UPS can trigger the accumulation of PrP^{C} (Hooper, 2003) in the ER, Golgi apparatus, and nucleus, where PrP^{C} is converted into PrP^{Sc} (Hooper, 2003; Ma and Lindquist, 2002; Ma et al., 2002). On the one hand, a study in PrP^{C} knockout mice indicates that transmitted PrP^{Sc} has no toxicity resulting from the absence of PrP^{C} (Bueler et al., 1993). However, the transgenic mice over-expressing normal PrP^{C} could generate PrP^{Sc} protein inclusions, leading to progressive cerebellar degeneration and ataxia (Ma et al., 2002). Besides the UPS, it has been demonstrated that autophagy is responsible for general synaptic loss in CJD brains (Sikorska et al., 2004), but there is no deficiency of autophagy reported yet.

1.5 TNFα pathway

Although tumor necrosis factor (TNF) was known for its anticancer activity more than a century ago, the human TNF cDNA was first cloned in 1984. Since then, studies have shown that TNF play a role in a number of biological processes including inflammation, proliferation, cell migration, apoptosis, and necrosis (Eissner et al., 2000; Eissner et al., 2004; Harashima et al., 2001; Ware, 2005). TNFα is synthesized as a monomeric transmembrane protein (tmTNF). It is then inserted into the plasma membrane as a

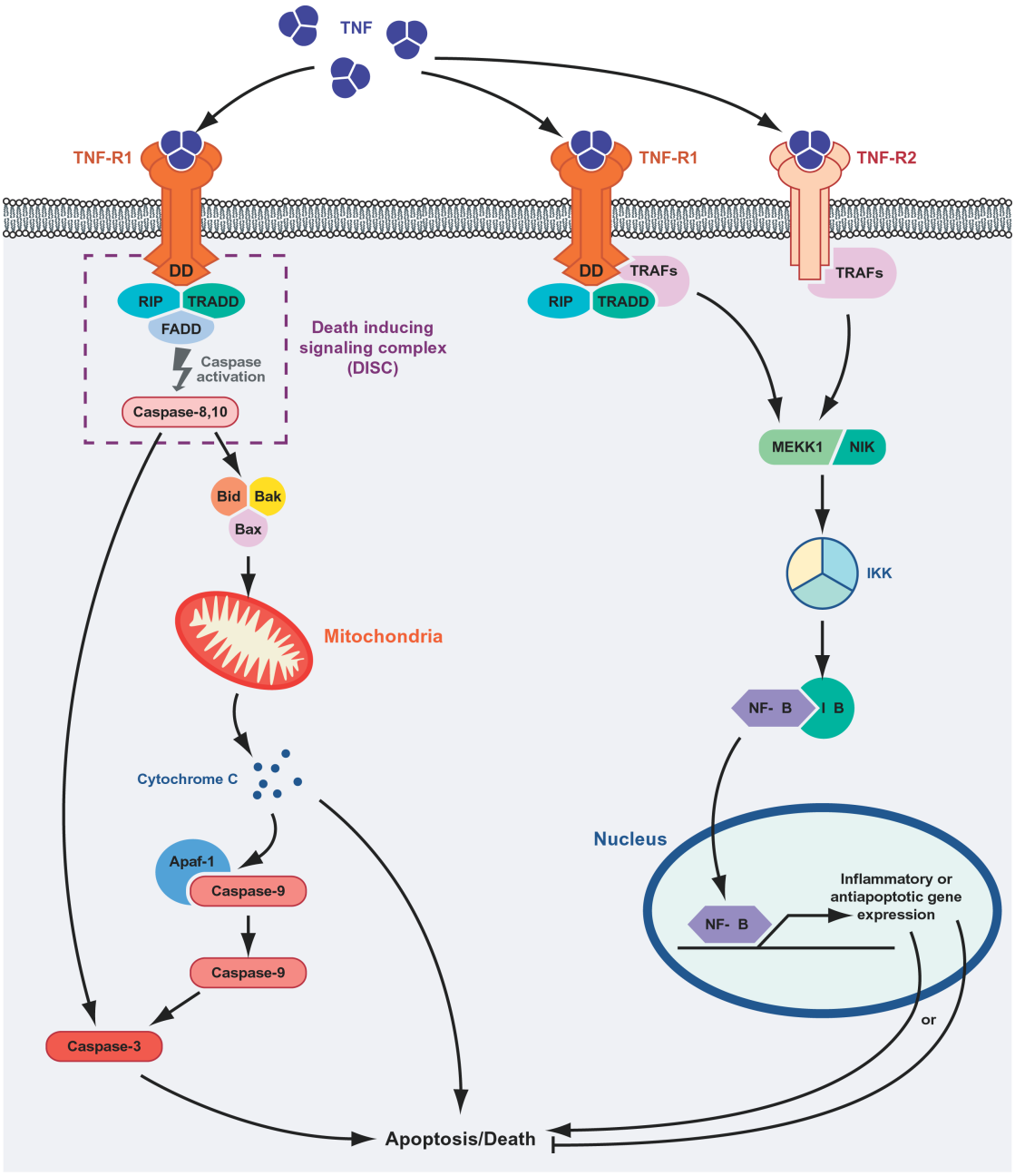
homotrimer, which is further modified by the matrix metalloprotease TNF alpha converting enzyme (TACE) to a soluble trimer (solTNF). TNFα can function in an autocrine or paracrine fashion. In the CNS, microglias, astrocytes and several types of neurons can produce tmTNF and solTNF (Chung et al., 2005; Lieberman et al., 1989; Morganti-Kossman et al., 1997). Studies have also shown that both tmTNF and solTNF are biologically active (Idriss and Naismith, 2000; MacEwan, 2002). In TNFα signaling, there are two distinct transmembrane receptors, TNF receptor-1 (TNF-R1, Tnfrsf1a) and TNF receptor-2 (TNF-R2, Tnfrsf1b). Although they specifically bind TNFα ligands, the two receptors differ in expression pattern, affinity for ligand, their structures of intracellular domains (Chan et al., 2000; Engelmann et al., 1990), and downstream signaling pathways, which give rise to their distinct responses to TNFα stimulation. For example, TNF-R2 is mainly expressed in the immune system and endothelial cells and it is activated by tmTNF preferentially (Grell, 1995; Grell et al., 1998). In contrast, TNF-R1 is widely expressed in numerous cell types and the receptor can be activated by tmTNF or solTNF. TNF-R1 activation by solTNF is thought to be the initiation of TNFα-mediated several cellular responses (Locksley et al., 2001) (Figure 1.7).

1.5.1 TNF-R1 signaling

In the beginning of TNF-R1 signaling, the extracellular domain of TNF-R1 binds to the TNFα trimer, which leads to the dissociation between silencer of death domains (SODD) and the intracellular domain of TNF-R1 (death domain, DD). The intracellular domain of TNF-R1 further interacts with an adaptor protein, TNF receptor-associated death domain (TRADD) (Tartaglia et al., 1993; Ware et al., 1996). In addition, TRADD recruits several proteins,

Figure 1.7 | TNF-mediated death and survival pathways are activated following interaction with the TNFRs.

The apoptotic pathway is activated through TNFR1 by forming the DISC, which activates caspase-8. Activated caspase-8 or −10 then activates the proapoptotic Bcl-2 family members, which leads to cell death by releasing cytochrome c from mitochondria and loss of MMP. The NF-κB-mediated survival pathway is activated by both TNF-R1 and TNF-R2. Association of TRAFs with these receptors activate signaling proteins like NIK (NF-κB inhibitor kinase) and MEKK1 (MAPK kinase 1), which activate the inhibitor of NF-κB (IkB) kinase (IKK) complex. IKK phosphorylates IkB, resulting in the degradation of the inhibitor. The free NF-κB than translocates to nucleus to induce the expression of inflammatory or anti-apoptotic genes. †From (Rahman and McFadden, 2006), courtesy of the author.


TNF
TNF-R1
TNF-R1
TNF-R2
DD
RIP
TRADD
FADD
Caspase activation
Caspase-8,10
Death inducing signaling complex (DISC)
DD
TRAFs
RIP
TRADD
TRAFs
MEKK1
NIK
IKK
Bid
Bak
Bax
Mitochondria
NF- B
I B
Cytochrome C
Nucleus
Apaf-1
Caspase-9
Caspase-9
NF- B
Inflammatory or antiapoptotic gene expression
or
Caspase-3
Apoptosis/Death

including receptor-interacting protein (RIP), TNF-R-associated factor 2 (TRAF2) (Hsu et al., 1996a; Hsu et al., 1996b; Hsu et al., 1995; Jiang et al., 1999), and Fas-associated death domain (FADD), to form a protein complex with TNF-R1. After the formation of the protein complex, TNF-R1 activates biological events by recruiting several signaling molecules, such as caspase-8, cellular inhibitor of apoptosis protein-1 (cIAP-1), cellular inhibitor of apoptosis protein-2 (cIAP-2), extracellular signal regulated kinase kinase kinase 1 (MEKK1), and apoptosis-stimulated kinase 1 (ASK-1).

The TNF-R1 protein complex can initiate cell survival signaling via TRAF2. TRAF2 activates MEKK1 and ASK1, ultimately resulting in phosphorylation of c-Jun NH2-terminal kinase (JNK). The activated JNK further phosphorylates c-Jun, increasing transcriptional activity of c-Jun. Interestingly, studies on TNF-induced JNK activation have shown that transient activation of JNK is TAK1-dependent and cytoprotective (Sato et al., 2005); however, sustained activation of JNK is ASK1 dependent and leads to caspase-dependent apoptosis (Tobiume et al., 2001). Some studies also documented that degradation of TRAF2 by cIAP-1 increases the sensitivity of cell to TNFα-induced apoptosis (Li et al., 2002). Additionally, cIAP-1 can also regulate apoptosis through mediating the ubiquitination and degradation of proapoptotic proteins, such as Smac/DIABLO (Hu and Yang, 2003). Other than cIAP-1-induced apoptosis, TNF-R1 can be internalized after associating with ligands, which leads to dissociation of the TRADD/TRAF2/RIP complex and association of FADD. The interaction of TNF-R1 with FADD further recruits caspase-8 to form the death-inducing signaling complex (DISC), which trigger the activation of caspase-8. The activation of caspase-8 further initiates an extrinsic protease-dependent apoptosis (Micheau and

Tschopp, 2003; Schneider-Brachert et al., 2004) as well as an intrinsic mitochondrial-induced apoptosis pathway by cleaving Bax and Bid (Gross et al., 1999; Wang et al., 2006; Zhao et al., 2001).

In the TNF-R1 pathway, RIP is known as another crucial molecule responsible for the activation of the transcription factor NF-κB to initiate pro-survival signaling, cellular proliferation, and cytokine production. The inactive form of NF-κB is normally associated with its inhibitor, inhibitor of κB (IκB), which retains NF-κB within the cytoplasm. Upon TNFα stimulation, RIP activates a multi-protein IκB kinase (IKK) complex (Ghosh and Karin, 2002), consisting of Cdc37, Hsp90, IKKα and IKKβ, and IKKγ (NEMO). Cdc37 and Hsp90 function as a shuttle to translocate the protein complex from the cytoplasm to the membrane where the catalytic subunits of IKK protein complex, IKKα and IKKβ, phosphorylate IκB (Chen et al., 2002). Once it is phosphorylated, IκB undergoes ubiquitination and degradation. Hence, NF-κB becomes active and translocates to the nucleus to initiate the transcriptional events. Finally, studies using transgenic mice have demonstrated that IKKβ is responsible for activation of NF-κB, and IKKγ plays a role in regulation of IKK complex activation upon TNFα stimulation (Ruocco et al., 2005).

More interestingly, there is a delicate cross talk between NF-κB and JNK signaling in the TNF-R1 pathway. TNFα-induced JNK activation becomes higher and sustained in NF-κB null cells. Conversely, NF-κB-activated genes generate proteins to inhibit the activation of JNK. In addition, activation of NF-κB leads to increased synthesis of its inhibitory molecules, including IkB and cIAPs. Therefore, cellular functions are fine-tuned by TNF-R1 pathway.

1.5.2 TNFα signaling in CNS

Studies have shown that TNF signaling regulates several functions within the brain, including injury-mediated activation of microglia and astrocytes, permeability of the blood brain barrier, febrile responses, glutamatergic transmission, as well as synaptic plasticity and scaling (Beattie et al., 2002; Leon, 2002; Merrill, 1991; Pickering et al., 2005; Sedgwick et al., 2000; Selmaj et al., 1990; Stellwagen et al., 2005; Stellwagen and Malenka, 2006). In the CNS, TNFα signaling actually presents a dual role in neurotoxicity and neuroprotection. Owed to the differential expression patterns of TNF receptors on neuronal and glial cells, the ultimate TNFα-triggered biological effect depends on the areas of the brain or the types of neuronal cells (Akassoglou et al., 2003; Dopp et al., 1997; Dziewulska and Mossakowski, 2003; Fontaine et al., 2002; Sairanen et al., 2001). For example, the activation of NF-ϰB is linked to TNFα-mediated neuroprotection (Albensi and Mattson, 2000; Barger et al., 1995; Kaltschmidt et al., 1999); the reduced activity of NF-ϰB is associated with neurotoxicity (Botchkina et al., 1999; Sriram et al., 2002). Studies have shown that TRAF2 and the stress response gene BRE (brain, and reproductive organ expressed) are involved in the regulation of NF-ϰB activation in TNF-R1 signaling (Gu et al., 1998). The cortical neurons derived from TNFα-overexpressed mice show activation of NFϰB, which protects cells from glutamate toxicity and increases neuronal survival (Marchetti et al., 2004). In addition, the TNF-R2-deficient neurons are shown to be more sensitive to both TNFα and glutamate-induced death (Marchetti et al., 2004). In contrast, TNFα-deficient mice exhibit accelerated maturation of the dentate gyrus area and shorter dendritic arbors in the hippocampus (Golan et al., 2004), indicating that TNFα regulates

hippocampal neuronal development. More interestingly, in many neurodegenerative disorders, such as AD (Alvarez et al., 2007; Fillit et al., 1991; Paganelli et al., 2002) and PD (Bessler et al., 1999; Boka et al., 1994; Hirsch et al., 1998; Mogi et al., 1994; Mogi et al., 2000; Nagatsu et al., 2000), the neuropathies of the diseases associate with increased solTNF in the brain. Hence, owed to the presence of TNFα at the place with neuronal damage, it may be useful to develop therapeutic treatments for acute and chronic neurodegenerative disorders by targeting TNFα.

1.5.3 TNF-R1 in Alzheimer's disease

In AD patients, TNFα is found within the amyloid plaques (Dickson, 1997) consistent with the findings from the transgenic Tg2576 mice over-expressing a mutated form of human amyloid precursor protein (APP) that high levels of TNFα are present in amyloid plaques (Mehlhorn et al., 2000; Munch et al., 2003; Sly et al., 2001). Furthermore, deletion of TNF-R1 in APP23 transgenic mice which overexpress $APP_{KM670/671NL}$ can reduce Aβ accumulation, activation of microglia, loss of neurons, and memory deficits (He et al., 2007).

1.5.4 TNF-R1 in Parkinson's disease

Similar to many other neurodegenerative diseases, the levels of TNFα and solTNF-R1 are increased in PD patients. The areas with higher levels of TNFα and solTNF-R1 also present more loss of dopaminergic neurons (Boka et al., 1994; Hirsch et al., 1998; Mogi et al., 1994; Mogi et al., 2000), consistent with the findings from *in vitro* (Clarke and Branton, 2002; Gayle et al., 2002; McGuire et al., 2001) and *in vivo* (Aloe and Fiore, 1997; Carvey et al., 2005)

studies showing neurotoxicity of TNFα to dopaminergic neurons. Taken collectively, the results suggest that TNF-R1 signaling plays a role in the progressive loss of neurons in PD.

1.6 Project Overview

After the first decade of its discovery, FIP200 has been shown to regulate various cellular functions, such as proliferation, spreading, migration, cell growth and size regulation, autophagy, and survival. However, little is known about the role of FIP200 in CNS. In the brain, neuronal cell proliferation, migration, and cell death are essential processes required for normal brain function. Therefore, my project has focused on the analysis of FIP200 functions in CNS by using *in vivo* mice genetics approaches to test whether FIP200 is required for neuronal cell survival and to discover the possible mechanisms by which FIP200 regulates neuronal functions.

To bypass embryonic lethality caused by total deletion of FIP200 and to investigate the role of FIP200 in CNS, I initially generated transgenic mice in which the ablation of FIP200 in CNS is mediated by Cre recombinase driven by the nestin promotor. In the Nestin-CKO transgenic mice, the deletion of FIP200 occurs in the neurons and glial cells. As detailed in Chapter 2, this conditional knockout of FIP200 led to several neuropathologies, including ubiquitin aggregates, vacuolization changes, reactive gliosis, axonal swelling and degeneration, and increased cell death. Interestingly, these mice developed cerebellar ataxia and their Purkinje cells were degenerated as well.

Therefore, to characterize whether the cerebellar ataxia is resulting from deletion of FIP200 in the Purkinje cells, I further generated the transgenic mice of which the deletion of FIP200 is mediated by Cre recombinase driven by

hGFAP promotor. As detailed in Chapter 3, these mice did not exhibit any degeneration of the Purkinje cells by 8 weeks. In addition, I generated the transgenic mice in which the deletion of FIP200 is mediated by Cre recombinase driven by L7 promotor. In the cerebellum of L7-CKO transgenic mice, the Purkinje cells are the only population without expression of FIP200. This conditional knockout of FIP200 led to similar neuropathologies as nestin-CKO mice, including ubiquitin aggregates, vacuolization changes, reactive astrogliosis, axonal swelling and degeneration, and increased the Purkinje cell death. The transgenic mice also developed ataxia. To further investigate the mechanism by which deletion of FIP200 leads to loss of the Purkinje cells, I generated two different transgenic mice by using a double knockout strategy. One is specific ablation of FIP200 and p53 in the Purkinje cells. However, the double conditional knockout mice still exhibit loss of Purkinje cells and ataxia. These results suggest that the Purkinje cell death is not through p53-mediated apoptosis. The other is double deletion of FIP200 and TNF-R1 in the Purkinje cells. The double conditional knockout mice rescued the loss of Purkinje cells and vacuolization changes. I also found that deletion of FIP200 led to dysfunction of mitochondria, releasing cytochrome c. Interestingly, inaction of TNFR-1 reduced cytochrome c release in the L7-CKO. Taken together, the data indicate that TNFR-1-FIP200 pathway is responsible for Purkinje cell survival.

REFERENCES

Abbi, S., and J.L. Guan. 2002. Focal adhesion kinase: protein interactions and cellular functions. *Histol Histopathol*. 17:1163-71.

Abbi, S., H. Ueda, C. Zheng, L.A. Cooper, J. Zhao, R. Christopher, and J.L. Guan. 2002. Regulation of focal adhesion kinase by a novel protein inhibitor FIP200. *Mol Biol Cell*. 13:3178-91.

Abou-Sleiman, P.M., M.M. Muqit, and N.W. Wood. 2006. Expanding insights of mitochondrial dysfunction in Parkinson's disease. *Nat Rev Neurosci*. 7:207-19.

Agochiya, M., V.G. Brunton, D.W. Owens, E.K. Parkinson, C. Paraskeva, W.N. Keith, and M.C. Frame. 1999. Increased dosage and amplification of the focal adhesion kinase gene in human cancer cells. *Oncogene*. 18:5646-53.

Akassoglou, K., E. Douni, J. Bauer, H. Lassmann, G. Kollias, and L. Probert. 2003. Exclusive tumor necrosis factor (TNF) signaling by the p75TNF receptor triggers inflammatory ischemia in the CNS of transgenic mice. *Proc Natl Acad Sci U S A*. 100:709-14.

Albensi, B.C., and M.P. Mattson. 2000. Evidence for the involvement of TNF and NF-kappaB in hippocampal synaptic plasticity. *Synapse*. 35:151-9.

Aloe, L., and M. Fiore. 1997. TNF-alpha expressed in the brain of transgenic mice lowers central tyroxine hydroxylase immunoreactivity and alters grooming behavior. *Neurosci Lett*. 238:65-8.

Altman, J. 1972a. Postnatal development of the cerebellar cortex in the rat. 3. Maturation of the components of the granular layer. *J Comp Neurol*. 145:465-513.

Altman, J. 1972b. Postnatal development of the cerebellar cortex in the rat. I. The external germinal layer and the transitional molecular layer. *J Comp Neurol*. 145:353-97.

Alvarez, A., R. Cacabelos, C. Sanpedro, M. Garcia-Fantini, and M. Aleixandre. 2007. Serum TNF-alpha levels are increased and correlate negatively with free IGF-I in Alzheimer disease. *Neurobiol Aging*. 28:533-6.

Alves-Rodrigues, A., L. Gregori, and M.E. Figueiredo-Pereira. 1998. Ubiquitin, cellular inclusions and their role in neurodegeneration. *Trends Neurosci*. 21:516-20.

Ambrose, C.M., M.P. Duyao, G. Barnes, G.P. Bates, C.S. Lin, J. Srinidhi, S. Baxendale, H. Hummerich, H. Lehrach, M. Altherr, and et al. 1994. Structure and expression of the Huntington's disease gene: evidence against simple inactivation due to an expanded CAG repeat. *Somat Cell Mol Genet*. 20:27-38.

Amenta, F., D. Cavalotta, M.E. Del Valle, M. Mancini, M. Sabbatini, J.M. Torres, and J.A. Vega. 1994. Calbindin D-28k immunoreactivity in the rat cerebellar cortex: age-related changes. *Neurosci Lett*. 178:131-4.

Apps, R., and M. Garwicz. 2005. Anatomical and physiological foundations of cerebellar information processing. *Nat Rev Neurosci*. 6:297-311.

Armstrong, C.L., and R. Hawkes. 2000. Pattern formation in the cerebellar cortex. *Biochem Cell Biol*. 78:551-62.

Arsenio Nunes, M.L., and C. Sotelo. 1985. Development of the spinocerebellar system in the postnatal rat. *J Comp Neurol*. 237:291-306.

Auld, K.L., and P.A. Silver. 2006. Transcriptional regulation by the proteasome as a mechanism for cellular protein homeostasis. *Cell Cycle*. 5:1503-5.

Avraham, H., S.Y. Park, K. Schinkmann, and S. Avraham. 2000. RAFTK/Pyk2-mediated cellular signalling. *Cell Signal*. 12:123-33.

Bamba, N., T. Chano, T. Taga, S. Ohta, Y. Takeuchi, and H. Okabe. 2004. Expression and regulation of RB1CC1 in developing murine and human tissues. *Int J Mol Med*. 14:583-7.

Barger, S.W., D. Horster, K. Furukawa, Y. Goodman, J. Krieglstein, and M.P. Mattson. 1995. Tumor necrosis factors alpha and beta protect neurons against amyloid beta-peptide toxicity: evidence for involvement of a kappa B-binding factor and attenuation of peroxide and Ca2+ accumulation. *Proc Natl Acad Sci U S A*. 92:9328-32.

Baurle, J., and U. Grusser-Cornehls. 1994. Axonal torpedoes in cerebellar Purkinje cells of two normal mouse strains during aging. *Acta Neuropathol*. 88:237-45.

Beattie, E.C., D. Stellwagen, W. Morishita, J.C. Bresnahan, B.K. Ha, M. Von Zastrow, M.S. Beattie, and R.C. Malenka. 2002. Control of synaptic strength by glial TNFalpha. *Science*. 295:2282-5.

Beg, A.A., W.C. Sha, R.T. Bronson, S. Ghosh, and D. Baltimore. 1995. Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature*. 376:167-70.

Bence, N.F., R.M. Sampat, and R.R. Kopito. 2001. Impairment of the ubiquitin-proteasome system by protein aggregation. *Science*. 292:1552-5.

Bessler, H., R. Djaldetti, H. Salman, M. Bergman, and M. Djaldetti. 1999. IL-1 beta, IL-2, IL-6 and TNF-alpha production by peripheral blood mononuclear cells from patients with Parkinson's disease. *Biomed Pharmacother*. 53:141-5.

Betarbet, R., T.B. Sherer, and J.T. Greenamyre. 2005. Ubiquitin-proteasome system and Parkinson's diseases. *Exp Neurol*. 191 Suppl 1:S17-27.

Bickford, P.C., T. Gould, L. Briederick, K. Chadman, A. Pollock, D. Young, B. Shukitt-Hale, and J. Joseph. 2000. Antioxidant-rich diets improve cerebellar physiology and motor learning in aged rats. *Brain Res*. 866:211-7.

Blandini, F., E. Sinforiani, C. Pacchetti, A. Samuele, E. Bazzini, R. Zangaglia, G. Nappi, and E. Martignoni. 2006. Peripheral proteasome and caspase activity in Parkinson disease and Alzheimer disease. *Neurology*. 66:529-34.

Boka, G., P. Anglade, D. Wallach, F. Javoy-Agid, Y. Agid, and E.C. Hirsch. 1994. Immunocytochemical analysis of tumor necrosis factor and its receptors in Parkinson's disease. *Neurosci Lett*. 172:151-4.

Botchkina, G.I., E. Geimonen, M.L. Bilof, O. Villarreal, and K.J. Tracey. 1999. Loss of NF-kappaB activity during cerebral ischemia and TNF cytotoxicity. *Mol Med*. 5:372-81.

Brown, D.R. 2002. Copper and prion diseases. *Biochem Soc Trans*. 30:742-5.

Brunk, U.T., and A. Terman. 2002. The mitochondrial-lysosomal axis theory of aging: accumulation of damaged mitochondria as a result of imperfect autophagocytosis. *Eur J Biochem*. 269:1996-2002.

Bueler, H., A. Aguzzi, A. Sailer, R.A. Greiner, P. Autenried, M. Aguet, and C. Weissmann. 1993. Mice devoid of PrP are resistant to scrapie. *Cell*. 73:1339-47.

Busch, A., S. Engemann, R. Lurz, H. Okazawa, H. Lehrach, and E.E. Wanker. 2003. Mutant huntingtin promotes the fibrillogenesis of wild-type

huntingtin: a potential mechanism for loss of huntingtin function in Huntington's disease. *J Biol Chem*. 278:41452-61.

Caddy, K.W., and T.J. Biscoe. 1979. Structural and quantitative studies on the normal C3H and Lurcher mutant mouse. *Philos Trans R Soc Lond B Biol Sci*. 287:167-201.

Carvey, P.M., E.Y. Chen, J.W. Lipton, C.W. Tong, Q.A. Chang, and Z.D. Ling. 2005. Intra-parenchymal injection of tumor necrosis factor-alpha and interleukin 1-beta produces dopamine neuron loss in the rat. *J Neural Transm*. 112:601-12.

Cataldo, A.M., J.L. Barnett, S.A. Berman, J. Li, S. Quarless, S. Bursztajn, C. Lippa, and R.A. Nixon. 1995. Gene expression and cellular content of cathepsin D in Alzheimer's disease brain: evidence for early up-regulation of the endosomal-lysosomal system. *Neuron*. 14:671-80.

Cha, J.H. 2000. Transcriptional dysregulation in Huntington's disease. *Trends Neurosci*. 23:387-92.

Chai, Y., S.L. Koppenhafer, S.J. Shoesmith, M.K. Perez, and H.L. Paulson. 1999. Evidence for proteasome involvement in polyglutamine disease: localization to nuclear inclusions in SCA3/MJD and suppression of polyglutamine aggregation in vitro. *Hum Mol Genet*. 8:673-82.

Chan, E.Y., S. Kir, and S.A. Tooze. 2007. siRNA screening of the kinome identifies ULK1 as a multidomain modulator of autophagy. *J Biol Chem*. 282:25464-74.

Chan, E.Y., A. Longatti, N.C. McKnight, and S.A. Tooze. 2009. Kinase-inactivated ULK proteins inhibit autophagy via their conserved C-terminal domains using an Atg13-independent mechanism. *Mol Cell Biol*. 29:157-71.

Chan, F.K., H.J. Chun, L. Zheng, R.M. Siegel, K.L. Bui, and M.J. Lenardo. 2000. A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. *Science*. 288:2351-4.

Chang, L.K., R.E. Schmidt, and E.M. Johnson, Jr. 2003. Alternating metabolic pathways in NGF-deprived sympathetic neurons affect caspase-independent death. *J Cell Biol*. 162:245-56.

Chano, T., S. Ikegawa, K. Kontani, H. Okabe, N. Baldini, and Y. Saeki. 2002a. Identification of RB1CC1, a novel human gene that can induce RB1 in various human cells. *Oncogene*. 21:1295-8.

Chano, T., S. Ikegawa, F. Saito-Ohara, J. Inazawa, A. Mabuchi, Y. Saeki, and H. Okabe. 2002b. Isolation, characterization and mapping of the mouse and human RB1CC1 genes. *Gene*. 291:29-34.

Chano, T., K. Kontani, K. Teramoto, H. Okabe, and S. Ikegawa. 2002c. Truncating mutations of RB1CC1 in human breast cancer. *Nat Genet*. 31:285-8.

Chano, T., H. Okabe, and C.M. Hulette. 2007. RB1CC1 insufficiency causes neuronal atrophy through mTOR signaling alteration and involved in the pathology of Alzheimer's diseases. *Brain Res*. 1168:97-105.

Chano, T., Y. Saeki, M. Serra, K. Matsumoto, and H. Okabe. 2002d. Preferential expression of RB1-inducible coiled-coil 1 in terminal differentiated musculoskeletal cells. *Am J Pathol*. 161:359-64.

Chano, T., M. Saji, H. Inoue, K. Minami, T. Kobayashi, O. Hino, and H. Okabe. 2006. Neuromuscular abundance of RB1CC1 contributes to the non-proliferating enlarged cell phenotype through both RB1 maintenance and TSC1 degradation. *Int J Mol Med*. 18:425-32.

Chedotal, A., E. Bloch-Gallego, and C. Sotelo. 1997. The embryonic cerebellum contains topographic cues that guide developing inferior olivary axons. *Development*. 124:861-70.

Chen, G., P. Cao, and D.V. Goeddel. 2002. TNF-induced recruitment and activation of the IKK complex require Cdc37 and Hsp90. *Mol Cell*. 9:401-10.

Christopher, R.A., and J.L. Guan. 2000. To move or not: how a cell responds (Review). *Int J Mol Med*. 5:575-81.

Chung, C.Y., H. Seo, K.C. Sonntag, A. Brooks, L. Lin, and O. Isacson. 2005. Cell type-specific gene expression of midbrain dopaminergic neurons reveals molecules involved in their vulnerability and protection. *Hum Mol Genet*. 14:1709-25.

Chung, K.K., Y. Zhang, K.L. Lim, Y. Tanaka, H. Huang, J. Gao, C.A. Ross, V.L. Dawson, and T.M. Dawson. 2001. Parkin ubiquitinates the alpha-synuclein-interacting protein, synphilin-1: implications for Lewy-body formation in Parkinson disease. *Nat Med*. 7:1144-50.

Clarke, D.J., and R.L. Branton. 2002. A role for tumor necrosis factor alpha in death of dopaminergic neurons following neural transplantation. *Exp Neurol*. 176:154-62.

Conway, K.A., S.J. Lee, J.C. Rochet, T.T. Ding, R.E. Williamson, and P.T. Lansbury, Jr. 2000. Acceleration of oligomerization, not fibrillization, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson's disease: implications for pathogenesis and therapy. *Proc Natl Acad Sci U S A*. 97:571-6.

Cuervo, A.M. 2004. Autophagy: in sickness and in health. *Trends Cell Biol*. 14:70-7.

Cuervo, A.M. 2006. Autophagy in neurons: it is not all about food. *Trends Mol Med*. 12:461-4.

Cummings, C.J., M.A. Mancini, B. Antalffy, D.B. DeFranco, H.T. Orr, and H.Y. Zoghbi. 1998. Chaperone suppression of aggregation and altered subcellular proteasome localization imply protein misfolding in SCA1. *Nat Genet*. 19:148-54.

Cummings, C.J., E. Reinstein, Y. Sun, B. Antalffy, Y. Jiang, A. Ciechanover, H.T. Orr, A.L. Beaudet, and H.Y. Zoghbi. 1999. Mutation of the E6-AP ubiquitin ligase reduces nuclear inclusion frequency while accelerating polyglutamine-induced pathology in SCA1 mice. *Neuron*. 24:879-92.

Davis, R.J. 2000. Signal transduction by the JNK group of MAP kinases. *Cell*. 103:239-52.

Dawson, T.M., and V.L. Dawson. 2003. Rare genetic mutations shed light on the pathogenesis of Parkinson disease. *J Clin Invest*. 111:145-51.

de Bilbao, F., E. Guarin, P. Nef, P. Vallet, P. Giannakopoulos, and M. Dubois-Dauphin. 1999. Postnatal distribution of cpp32/caspase 3 mRNA in the mouse central nervous system: an in situ hybridization study. *J Comp Neurol*. 409:339-57.

de Vrij, F.M., D.F. Fischer, F.W. van Leeuwen, and E.M. Hol. 2004. Protein quality control in Alzheimer's disease by the ubiquitin proteasome system. *Prog Neurobiol*. 74:249-70.

Dedeoglu, A., J.K. Kubilus, T.M. Jeitner, S.A. Matson, M. Bogdanov, N.W. Kowall, W.R. Matson, A.J. Cooper, R.R. Ratan, M.F. Beal, S.M. Hersch, and R.J. Ferrante. 2002. Therapeutic effects of cystamine in a murine model of Huntington's disease. *J Neurosci*. 22:8942-50.

Desclin, J.C. 1974. Histological evidence supporting the inferior olive as the major source of cerebellar climbing fibers in the rat. *Brain Res*. 77:365-84.

Dev, K.K., H. van der Putten, B. Sommer, and G. Rovelli. 2003. Part I: parkin-associated proteins and Parkinson's disease. *Neuropharmacology*. 45:1-13.

Dickson, D.W. 1997. The pathogenesis of senile plaques. *J Neuropathol Exp Neurol*. 56:321-39.

Ding, Q., E. Dimayuga, S. Martin, A.J. Bruce-Keller, V. Nukala, A.M. Cuervo, and J.N. Keller. 2003. Characterization of chronic low-level proteasome inhibition on neural homeostasis. *J Neurochem*. 86:489-97.

Ding, Q., J.J. Lewis, K.M. Strum, E. Dimayuga, A.J. Bruce-Keller, J.C. Dunn, and J.N. Keller. 2002. Polyglutamine expansion, protein aggregation, proteasome activity, and neural survival. *J Biol Chem*. 277:13935-42.

Donohue, T.M., Jr. 2002. The ubiquitin-proteasome system and its role in ethanol-induced disorders. *Addict Biol*. 7:15-28.

Dopp, J.M., A. Mackenzie-Graham, G.C. Otero, and J.E. Merrill. 1997. Differential expression, cytokine modulation, and specific functions of type-1 and type-2 tumor necrosis factor receptors in rat glia. *J Neuroimmunol*. 75:104-12.

Doskeland, A.P., and T. Flatmark. 2002. Ubiquitination of soluble and membrane-bound tyrosine hydroxylase and degradation of the soluble form. *Eur J Biochem*. 269:1561-9.

Doss-Pepe, E.W., E.S. Stenroos, W.G. Johnson, and K. Madura. 2003. Ataxin-3 interactions with rad23 and valosin-containing protein and its associations with ubiquitin chains and the proteasome are consistent with a role in ubiquitin-mediated proteolysis. *Mol Cell Biol*. 23:6469-83.

Dulabon, L., E.C. Olson, M.G. Taglienti, S. Eisenhuth, B. McGrath, C.A. Walsh, J.A. Kreidberg, and E.S. Anton. 2000. Reelin binds alpha3beta1 integrin and inhibits neuronal migration. *Neuron*. 27:33-44.

Dziewulska, D., and M.J. Mossakowski. 2003. Cellular expression of tumor necrosis factor a and its receptors in human ischemic stroke. *Clin Neuropathol*. 22:35-40.

Edwards, M.A., M. Yamamoto, and V.S. Caviness, Jr. 1990. Organization of radial glia and related cells in the developing murine CNS. An analysis based upon a new monoclonal antibody marker. *Neuroscience*. 36:121-44.

Eissner, G., S. Kirchner, H. Lindner, W. Kolch, P. Janosch, M. Grell, P. Scheurich, R. Andreesen, and E. Holler. 2000. Reverse signaling through transmembrane TNF confers resistance to lipopolysaccharide in human monocytes and macrophages. *J Immunol*. 164:6193-8.

Eissner, G., W. Kolch, and P. Scheurich. 2004. Ligands working as receptors: reverse signaling by members of the TNF superfamily enhance the plasticity of the immune system. *Cytokine Growth Factor Rev*. 15:353-66.

Elsasser, S., and D. Finley. 2005. Delivery of ubiquitinated substrates to protein-unfolding machines. *Nat Cell Biol*. 7:742-9.

Engelmann, H., H. Holtmann, C. Brakebusch, Y.S. Avni, I. Sarov, Y. Nophar, E. Hadas, O. Leitner, and D. Wallach. 1990. Antibodies to a soluble form of a tumor necrosis factor (TNF) receptor have TNF-like activity. *J Biol Chem*. 265:14497-504.

Fabunmi, R.P., W.C. Wigley, P.J. Thomas, and G.N. DeMartino. 2000. Activity and regulation of the centrosome-associated proteasome. *J Biol Chem*. 275:409-13.

Fan, H., M. Favero, and M.W. Vogel. 2001. Elimination of Bax expression in mice increases cerebellar purkinje cell numbers but not the number of granule cells. *J Comp Neurol*. 436:82-91.

Fattoretti, P., C. Bertoni-Freddari, U. Caselli, R. Paoloni, and W. Meier-Ruge. 1998. Impaired succinic dehydrogenase activity of rat Purkinje cell mitochondria during aging. *Mech Ageing Dev*. 101:175-82.

Fillit, H., W.H. Ding, L. Buee, J. Kalman, L. Altstiel, B. Lawlor, and G. Wolf-Klein. 1991. Elevated circulating tumor necrosis factor levels in Alzheimer's disease. *Neurosci Lett*. 129:318-20.

Fontaine, V., S. Mohand-Said, N. Hanoteau, C. Fuchs, K. Pfizenmaier, and U. Eisel. 2002. Neurodegenerative and neuroprotective effects of tumor Necrosis factor (TNF) in retinal ischemia: opposite roles of TNF receptor 1 and TNF receptor 2. *J Neurosci*. 22:RC216.

Frankowski, H., M. Missotten, P.A. Fernandez, I. Martinou, P. Michel, R. Sadoul, and J.C. Martinou. 1995. Function and expression of the Bcl-x gene in the developing and adult nervous system. *Neuroreport*. 6:1917-21.

Gallagher, E., B.W. Howell, P. Soriano, J.A. Cooper, and R. Hawkes. 1998. Cerebellar abnormalities in the disabled (mdab1-1) mouse. *J Comp Neurol*. 402:238-51.

Gan, B., Z.K. Melkoumian, X. Wu, K.L. Guan, and J.L. Guan. 2005. Identification of FIP200 interaction with the TSC1-TSC2 complex and its role in regulation of cell size control. *J Cell Biol*. 170:379-89.

Gan, B., X. Peng, T. Nagy, A. Alcaraz, H. Gu, and J.L. Guan. 2006. Role of FIP200 in cardiac and liver development and its regulation of TNFalpha and TSC-mTOR signaling pathways. *J Cell Biol*. 175:121-33.

Ganley, I.G., D.H. Lam, J. Wang, X. Ding, S. Chen, and X. Jiang. 2009. ULK1-ATG13-FIP200 complex mediates mTOR signaling and is essential for autophagy. *J Biol Chem*.

Gao, M., and M. Karin. 2005. Regulating the regulators: control of protein ubiquitination and ubiquitin-like modifications by extracellular stimuli. *Mol Cell*. 19:581-93.

Gayle, D.A., Z. Ling, C. Tong, T. Landers, J.W. Lipton, and P.M. Carvey. 2002. Lipopolysaccharide (LPS)-induced dopamine cell loss in culture: roles of tumor necrosis factor-alpha, interleukin-1beta, and nitric oxide. *Brain Res Dev Brain Res*. 133:27-35.

Ghetti, B., J. Norton, and L.C. Triarhou. 1987. Nerve cell atrophy and loss in the inferior olivary complex of "Purkinje cell degeneration" mutant mice. *J Comp Neurol*. 260:409-22.

Ghosh, S., and M. Karin. 2002. Missing pieces in the NF-kappaB puzzle. *Cell*. 109 Suppl:S81-96.

Gidalevitz, T., A. Ben-Zvi, K.H. Ho, H.R. Brignull, and R.I. Morimoto. 2006. Progressive disruption of cellular protein folding in models of polyglutamine diseases. *Science*. 311:1471-4.

Gilmore, E.C., and K. Herrup. 2000. Cortical development: receiving reelin. *Curr Biol*. 10:R162-6.

Glatzel, M., K. Stoeck, H. Seeger, T. Luhrs, and A. Aguzzi. 2005. Human prion diseases: molecular and clinical aspects. *Arch Neurol*. 62:545-52.

Golan, H., T. Levav, A. Mendelsohn, and M. Huleihel. 2004. Involvement of tumor necrosis factor alpha in hippocampal development and function. *Cereb Cortex*. 14:97-105.

Goldberg, A.L. 2003. Protein degradation and protection against misfolded or damaged proteins. *Nature*. 426:895-9.

Goldowitz, D., and K. Hamre. 1998. The cells and molecules that make a cerebellum. *Trends Neurosci*. 21:375-82.

Gould, B.B. 1979. The organization of afferents to the cerebellar cortex in the cat: projections from the deep cerebellar nuclei. *J Comp Neurol*. 184:27-42.

Greene, J.C., A.J. Whitworth, I. Kuo, L.A. Andrews, M.B. Feany, and L.J. Pallanck. 2003. Mitochondrial pathology and apoptotic muscle degeneration in Drosophila parkin mutants. *Proc Natl Acad Sci U S A*. 100:4078-83.

Grell, M. 1995. Tumor necrosis factor (TNF) receptors in cellular signaling of soluble and membrane-expressed TNF. *J Inflamm*. 47:8-17.

Grell, M., H. Wajant, G. Zimmermann, and P. Scheurich. 1998. The type 1 receptor (CD120a) is the high-affinity receptor for soluble tumor necrosis factor. *Proc Natl Acad Sci U S A*. 95:570-5.

Grishkat, H.L., and L.M. Eisenman. 1995. Development of the spinocerebellar projection in the prenatal mouse. *J Comp Neurol*. 363:93-108.

Gross, A., X.M. Yin, K. Wang, M.C. Wei, J. Jockel, C. Milliman, H. Erdjument-Bromage, P. Tempst, and S.J. Korsmeyer. 1999. Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. *J Biol Chem*. 274:1156-63.

Gu, C., A. Castellino, J.Y. Chan, and M.V. Chao. 1998. BRE: a modulator of TNF-alpha action. *FASEB J*. 12:1101-8.

Gusella, J.F., and M.E. MacDonald. 2000. Molecular genetics: unmasking polyglutamine triggers in neurodegenerative disease. *Nat Rev Neurosci*. 1:109-15.

Hara, T., K. Nakamura, M. Matsui, A. Yamamoto, Y. Nakahara, R. Suzuki-Migishima, M. Yokoyama, K. Mishima, I. Saito, H. Okano, and N. Mizushima. 2006. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature*. 441:885-9.

Hara, T., A. Takamura, C. Kishi, S. Iemura, T. Natsume, J.L. Guan, and N. Mizushima. 2008. FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells. *J Cell Biol*. 181:497-510.

Harashima, S., T. Horiuchi, N. Hatta, C. Morita, M. Higuchi, T. Sawabe, H. Tsukamoto, T. Tahira, K. Hayashi, S. Fujita, and Y. Niho. 2001. Outside-to-inside signal through the membrane TNF-alpha induces E-selectin (CD62E) expression on activated human CD4+ T cells. *J Immunol*. 166:130-6.

Hardy, J., and D.J. Selkoe. 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science*. 297:353-6.

Hawkes, R., and L.M. Eisenman. 1997. Stripes and zones: the origins of regionalization of the adult cerebellum. *Perspect Dev Neurobiol*. 5:95-105.

He, P., Z. Zhong, K. Lindholm, L. Berning, W. Lee, C. Lemere, M. Staufenbiel, R. Li, and Y. Shen. 2007. Deletion of tumor necrosis factor death receptor inhibits amyloid beta generation and prevents learning and memory deficits in Alzheimer's mice. *J Cell Biol*. 178:829-41.

Herrup, K., and B. Kuemerle. 1997. The compartmentalization of the cerebellum. *Annu Rev Neurosci*. 20:61-90.

Hershko, A. 2005. The ubiquitin system for protein degradation and some of its roles in the control of the cell-division cycle (Nobel lecture). *Angew Chem Int Ed Engl*. 44:5932-43.

Hicke, L., H.L. Schubert, and C.P. Hill. 2005. Ubiquitin-binding domains. *Nat Rev Mol Cell Biol*. 6:610-21.

Hilberg, F., A. Aguzzi, N. Howells, and E.F. Wagner. 1993. c-jun is essential for normal mouse development and hepatogenesis. *Nature*. 365:179-81.

Hirsch, E.C., S. Hunot, P. Damier, and B. Faucheux. 1998. Glial cells and inflammation in Parkinson's disease: a role in neurodegeneration? *Ann Neurol*. 44:S115-20.

Hoeflich, K.P., J. Luo, E.A. Rubie, M.S. Tsao, O. Jin, and J.R. Woodgett. 2000. Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. *Nature*. 406:86-90.

Hoffman, E.K., H.M. Wilcox, R.W. Scott, and R. Siman. 1996. Proteasome inhibition enhances the stability of mouse Cu/Zn superoxide dismutase with mutations linked to familial amyotrophic lateral sclerosis. *J Neurol Sci*. 139:15-20.

Hoffner, G., P. Kahlem, and P. Djian. 2002. Perinuclear localization of huntingtin as a consequence of its binding to microtubules through an interaction with beta-tubulin: relevance to Huntington's disease. *J Cell Sci*. 115:941-8.

Hooper, N.M. 2003. Could inhibition of the proteasome cause mad cow disease? *Trends Biotechnol*. 21:144-5.

Hosokawa, N., T. Hara, T. Kaizuka, C. Kishi, A. Takamura, Y. Miura, S.I. Iemura, T. Natsume, K. Takehana, N. Yamada, J.L. Guan, N. Oshiro, and N. Mizushima. 2009. Nutrient-dependent mTORC1 Association with

the ULK1-Atg13-FIP200 Complex Required for Autophagy. *Mol Biol Cell*.

Howell, B.W., R. Hawkes, P. Soriano, and J.A. Cooper. 1997. Neuronal position in the developing brain is regulated by mouse disabled-1. *Nature*. 389:733-7.

Hsu, H., J. Huang, H.B. Shu, V. Baichwal, and D.V. Goeddel. 1996a. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. *Immunity*. 4:387-96.

Hsu, H., H.B. Shu, M.G. Pan, and D.V. Goeddel. 1996b. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell*. 84:299-308.

Hsu, H., J. Xiong, and D.V. Goeddel. 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell*. 81:495-504.

Hu, S., and X. Yang. 2003. Cellular inhibitor of apoptosis 1 and 2 are ubiquitin ligases for the apoptosis inducer Smac/DIABLO. *J Biol Chem*. 278:10055-60.

Hyun, D.H., M. Lee, B. Halliwell, and P. Jenner. 2003. Proteasomal inhibition causes the formation of protein aggregates containing a wide range of proteins, including nitrated proteins. *J Neurochem*. 86:363-73.

Idriss, H.T., and J.H. Naismith. 2000. TNF alpha and the TNF receptor superfamily: structure-function relationship(s). *Microsc Res Tech*. 50:184-95.

Imai, Y., M. Soda, S. Hatakeyama, T. Akagi, T. Hashikawa, K.I. Nakayama, and R. Takahashi. 2002. CHIP is associated with Parkin, a gene responsible

for familial Parkinson's disease, and enhances its ubiquitin ligase activity. *Mol Cell*. 10:55-67.

Imai, Y., M. Soda, H. Inoue, N. Hattori, Y. Mizuno, and R. Takahashi. 2001. An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. *Cell*. 105:891-902.

Imai, Y., M. Soda, and R. Takahashi. 2000. Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. *J Biol Chem*. 275:35661-4.

Ironside, J.W., L. McCardle, P.A. Hayward, and J.E. Bell. 1993. Ubiquitin immunocytochemistry in human spongiform encephalopathies. *Neuropathol Appl Neurobiol*. 19:134-40.

Ishigaki, S., J. Niwa, Y. Ando, T. Yoshihara, K. Sawada, M. Doyu, M. Yamamoto, K. Kato, Y. Yotsumoto, and G. Sobue. 2002. Differentially expressed genes in sporadic amyotrophic lateral sclerosis spinal cords--screening by molecular indexing and subsequent cDNA microarray analysis. *FEBS Lett*. 531:354-8.

Ito, M. 1984. The cerebellum and neural control. Raven Press, New York. xvii, 580 p.

Ito, M., and M. Yoshida. 1964. The cerebellar-evoked monosynaptic inhibition of Deiters' neurones. *Experientia*. 20:515-6.

Iwata, A., J.C. Christianson, M. Bucci, L.M. Ellerby, N. Nukina, L.S. Forno, and R.R. Kopito. 2005a. Increased susceptibility of cytoplasmic over nuclear polyglutamine aggregates to autophagic degradation. *Proc Natl Acad Sci U S A*. 102:13135-40.

Iwata, A., B.E. Riley, J.A. Johnston, and R.R. Kopito. 2005b. HDAC6 and microtubules are required for autophagic degradation of aggregated huntingtin. *J Biol Chem*. 280:40282-92.

Jana, N.R., E.A. Zemskov, G. Wang, and N. Nukina. 2001. Altered proteasomal function due to the expression of polyglutamine-expanded truncated N-terminal huntingtin induces apoptosis by caspase activation through mitochondrial cytochrome c release. *Hum Mol Genet*. 10:1049-59.

Ji, Z., and R. Hawkes. 1995. Developing mossy fiber terminal fields in the rat cerebellar cortex may segregate because of Purkinje cell compartmentation and not competition. *J Comp Neurol*. 359:197-212.

Jiang, Y., J.D. Woronicz, W. Liu, and D.V. Goeddel. 1999. Prevention of constitutive TNF receptor 1 signaling by silencer of death domains. *Science*. 283:543-6.

Jin, T., Y. Gu, G. Zanusso, M. Sy, A. Kumar, M. Cohen, P. Gambetti, and N. Singh. 2000. The chaperone protein BiP binds to a mutant prion protein and mediates its degradation by the proteasome. *J Biol Chem*. 275:38699-704.

Johnson, R.T. 2005. Prion diseases. *Lancet Neurol*. 4:635-42.

Johnston, J.A., M.J. Dalton, M.E. Gurney, and R.R. Kopito. 2000. Formation of high molecular weight complexes of mutant Cu, Zn-superoxide dismutase in a mouse model for familial amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A*. 97:12571-6.

Johnston, J.A., C.L. Ward, and R.R. Kopito. 1998. Aggresomes: a cellular response to misfolded proteins. *J Cell Biol*. 143:1883-98.

Jones, R.J., V.G. Brunton, and M.C. Frame. 2000. Adhesion-linked kinases in cancer; emphasis on src, focal adhesion kinase and PI 3-kinase. *Eur J Cancer*. 36:1595-606.

Jung, C.H., C.B. Jun, S.H. Ro, Y.M. Kim, N.M. Otto, J. Cao, M. Kundu, and D.H. Kim. 2009. ULK-Atg13-FIP200 Complexes Mediate mTOR Signaling to the Autophagy Machinery. *Mol Biol Cell*.

Kahlem, P., C. Terre, H. Green, and P. Djian. 1996. Peptides containing glutamine repeats as substrates for transglutaminase-catalyzed cross-linking: relevance to diseases of the nervous system. *Proc Natl Acad Sci U S A*. 93:14580-5.

Kalchman, M.A., R.K. Graham, G. Xia, H.B. Koide, J.G. Hodgson, K.C. Graham, Y.P. Goldberg, R.D. Gietz, C.M. Pickart, and M.R. Hayden. 1996. Huntingtin is ubiquitinated and interacts with a specific ubiquitin-conjugating enzyme. *J Biol Chem*. 271:19385-94.

Kaltschmidt, B., M. Uherek, H. Wellmann, B. Volk, and C. Kaltschmidt. 1999. Inhibition of NF-kappaB potentiates amyloid beta-mediated neuronal apoptosis. *Proc Natl Acad Sci U S A*. 96:9409-14.

Kandel, E.R., J.H. Schwartz, and T.M. Jessell. 2000. Principles of neural science. McGraw-Hill, Health Professions Division, New York. xli, 1414 p. pp.

Karpuj, M.V., M.W. Becher, J.E. Springer, D. Chabas, S. Youssef, R. Pedotti, D. Mitchell, and L. Steinman. 2002. Prolonged survival and decreased abnormal movements in transgenic model of Huntington disease, with administration of the transglutaminase inhibitor cystamine. *Nat Med*. 8:143-9.

Kegel, K.B., M. Kim, E. Sapp, C. McIntyre, J.G. Castano, N. Aronin, and M. DiFiglia. 2000. Huntingtin expression stimulates endosomal-lysosomal activity, endosome tubulation, and autophagy. *J Neurosci*. 20:7268-78.

Keller, J.N., K.B. Hanni, and W.R. Markesbery. 2000. Impaired proteasome function in Alzheimer's disease. *J Neurochem*. 75:436-9.

Kitada, T., S. Asakawa, N. Hattori, H. Matsumine, Y. Yamamura, S. Minoshima, M. Yokochi, Y. Mizuno, and N. Shimizu. 1998. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature*. 392:605-8.

Kitao, Y., K. Hashimoto, T. Matsuyama, H. Iso, T. Tamatani, O. Hori, D.M. Stern, M. Kano, K. Ozawa, and S. Ogawa. 2004. ORP150/HSP12A regulates Purkinje cell survival: a role for endoplasmic reticulum stress in cerebellar development. *J Neurosci*. 24:1486-96.

Klionsky, D.J. 2006. Neurodegeneration: good riddance to bad rubbish. *Nature*. 441:819-20.

Kobayashi, T., O. Minowa, J. Kuno, H. Mitani, O. Hino, and T. Noda. 1999. Renal carcinogenesis, hepatic hemangiomatosis, and embryonic lethality caused by a germ-line Tsc2 mutation in mice. *Cancer Res*. 59:1206-11.

Kobayashi, T., O. Minowa, Y. Sugitani, S. Takai, H. Mitani, E. Kobayashi, T. Noda, and O. Hino. 2001. A germ-line Tsc1 mutation causes tumor development and embryonic lethality that are similar, but not identical to, those caused by Tsc2 mutation in mice. *Proc Natl Acad Sci U S A*. 98:8762-7.

Komatsu, M., S. Waguri, T. Chiba, S. Murata, J. Iwata, I. Tanida, T. Ueno, M. Koike, Y. Uchiyama, E. Kominami, and K. Tanaka. 2006. Loss of

autophagy in the central nervous system causes neurodegeneration in mice. *Nature*. 441:880-4.

Kontani, K., T. Chano, Y. Ozaki, N. Tezuka, S. Sawai, S. Fujino, Y. Saeki, and H. Okabe. 2003. RB1CC1 suppresses cell cycle progression through RB1 expression in human neoplastic cells. *Int J Mol Med*. 12:767-9.

Kopito, R.R. 2000. Aggresomes, inclusion bodies and protein aggregation. *Trends Cell Biol*. 10:524-30.

Laine, J., and H. Axelrad. 1994. The candelabrum cell: a new interneuron in the cerebellar cortex. *J Comp Neurol*. 339:159-73.

Laine, J., and H. Axelrad. 1998. Lugaro cells target basket and stellate cells in the cerebellar cortex. *Neuroreport*. 9:2399-403.

Lampert, P.W., D.C. Gajdusek, and C.J. Gibbs, Jr. 1972. Subacute spongiform virus encephalopathies. Scrapie, Kuru and Creutzfeldt-Jakob disease: a review. *Am J Pathol*. 68:626-52.

Landles, C., and G.P. Bates. 2004. Huntingtin and the molecular pathogenesis of Huntington's disease. Fourth in molecular medicine review series. *EMBO Rep*. 5:958-63.

Lang-Rollin, I.C., H.J. Rideout, M. Noticewala, and L. Stefanis. 2003. Mechanisms of caspase-independent neuronal death: energy depletion and free radical generation. *J Neurosci*. 23:11015-25.

Larsen, K.E., and D. Sulzer. 2002. Autophagy in neurons: a review. *Histol Histopathol*. 17:897-908.

Lauffenburger, D.A., and A.F. Horwitz. 1996. Cell migration: a physically integrated molecular process. *Cell*. 84:359-69.

Leclerc, N., C. Gravel, and R. Hawkes. 1988. Development of parasagittal zonation in the rat cerebellar cortex: MabQ113 antigenic bands are

created postnatally by the suppression of antigen expression in a subset of Purkinje cells. *J Comp Neurol*. 273:399-420.

Lee, J.P., C. Gerin, V.P. Bindokas, R. Miller, G. Ghadge, and R.P. Roos. 2002. No correlation between aggregates of Cu/Zn superoxide dismutase and cell death in familial amyotrophic lateral sclerosis. *J Neurochem*. 82:1229-38.

Lee, M., D. Hyun, B. Halliwell, and P. Jenner. 2001. Effect of the overexpression of wild-type or mutant alpha-synuclein on cell susceptibility to insult. *J Neurochem*. 76:998-1009.

Lee, S.Y., A. Reichlin, A. Santana, K.A. Sokol, M.C. Nussenzweig, and Y. Choi. 1997. TRAF2 is essential for JNK but not NF-kappaB activation and regulates lymphocyte proliferation and survival. *Immunity*. 7:703-13.

Leon, L.R. 2002. Invited review: cytokine regulation of fever: studies using gene knockout mice. *J Appl Physiol*. 92:2648-55.

Levine, B., and J. Yuan. 2005. Autophagy in cell death: an innocent convict? *J Clin Invest*. 115:2679-88.

Li, Q., D. Van Antwerp, F. Mercurio, K.F. Lee, and I.M. Verma. 1999. Severe liver degeneration in mice lacking the IkappaB kinase 2 gene. *Science*. 284:321-5.

Li, S.H., and X.J. Li. 2004. Huntingtin-protein interactions and the pathogenesis of Huntington's disease. *Trends Genet*. 20:146-54.

Li, X., Y. Yang, and J.D. Ashwell. 2002. TNF-RII and c-IAP1 mediate ubiquitination and degradation of TRAF2. *Nature*. 416:345-7.

Lieberman, A.P., P.M. Pitha, H.S. Shin, and M.L. Shin. 1989. Production of tumor necrosis factor and other cytokines by astrocytes stimulated with

lipopolysaccharide or a neurotropic virus. *Proc Natl Acad Sci U S A*. 86:6348-52.

Llinás, R.R., and American Medical Association Education and Research Foundation. Institute for Biomedical Research. 1969. Neurobiology of cerebellar evolution and development : proceedings of the First International Symposium of the Institute for Biomedical Research, American Medical Association/Education & Research Foundation. American Medical Association, [Chicago. x, 931 p. pp.

Locksley, R.M., N. Killeen, and M.J. Lenardo. 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell*. 104:487-501.

Lopez Salon, M., L. Morelli, E.M. Castano, E.F. Soto, and J.M. Pasquini. 2000. Defective ubiquitination of cerebral proteins in Alzheimer's disease. *J Neurosci Res*. 62:302-10.

Lopez Salon, M., L. Pasquini, M. Besio Moreno, J.M. Pasquini, and E. Soto. 2003. Relationship between beta-amyloid degradation and the 26S proteasome in neural cells. *Exp Neurol*. 180:131-43.

Lucking, C.B., A. Durr, V. Bonifati, J. Vaughan, G. De Michele, T. Gasser, B.S. Harhangi, G. Meco, P. Denefle, N.W. Wood, Y. Agid, and A. Brice. 2000. Association between early-onset Parkinson's disease and mutations in the parkin gene. *N Engl J Med*. 342:1560-7.

Lue, L.F., Y.M. Kuo, A.E. Roher, L. Brachova, Y. Shen, L. Sue, T. Beach, J.H. Kurth, R.E. Rydel, and J. Rogers. 1999. Soluble amyloid beta peptide concentration as a predictor of synaptic change in Alzheimer's disease. *Am J Pathol*. 155:853-62.

Lunkes, A., K.S. Lindenberg, L. Ben-Haiem, C. Weber, D. Devys, G.B. Landwehrmeyer, J.L. Mandel, and Y. Trottier. 2002. Proteases acting on

mutant huntingtin generate cleaved products that differentially build up cytoplasmic and nuclear inclusions. *Mol Cell*. 10:259-69.

Ma, J., and S. Lindquist. 2002. Conversion of PrP to a self-perpetuating PrPSc-like conformation in the cytosol. *Science*. 298:1785-8.

Ma, J., R. Wollmann, and S. Lindquist. 2002. Neurotoxicity and neurodegeneration when PrP accumulates in the cytosol. *Science*. 298:1781-5.

MacEwan, D.J. 2002. TNF receptor subtype signalling: differences and cellular consequences. *Cell Signal*. 14:477-92.

Madalosso, S.H., E.M. Perez-Villegas, and J.A. Armengol. 2005. Naturally occurring neuronal death during the postnatal development of Purkinje cells and their precerebellar afferent projections. *Brain Res Brain Res Rev*. 49:267-79.

Manto, M.-U., and M. Pandolfo. 2002. The cerebellum and its disorders. Cambridge University Press, Cambridge; New York. xxii, 589 p. pp.

Marchetti, L., M. Klein, K. Schlett, K. Pfizenmaier, and U.L. Eisel. 2004. Tumor necrosis factor (TNF)-mediated neuroprotection against glutamate-induced excitotoxicity is enhanced by N-methyl-D-aspartate receptor activation. Essential role of a TNF receptor 2-mediated phosphatidylinositol 3-kinase-dependent NF-kappa B pathway. *J Biol Chem*. 279:32869-81.

Marin-Teva, J.L., I. Dusart, C. Colin, A. Gervais, N. van Rooijen, and M. Mallat. 2004. Microglia promote the death of developing Purkinje cells. *Neuron*. 41:535-47.

Massey, A.C., S. Kaushik, G. Sovak, R. Kiffin, and A.M. Cuervo. 2006. Consequences of the selective blockage of chaperone-mediated autophagy. *Proc Natl Acad Sci U S A*. 103:5805-10.

Masters, C.L., and E.P. Richardson, Jr. 1978. Subacute spongiform encephalopathy (Creutzfeldt-Jakob disease). The nature and progression of spongiform change. *Brain*. 101:333-44.

Matilla, A., C. Gorbea, D.D. Einum, J. Townsend, A. Michalik, C. van Broeckhoven, C.C. Jensen, K.J. Murphy, L.J. Ptacek, and Y.H. Fu. 2001. Association of ataxin-7 with the proteasome subunit S4 of the 19S regulatory complex. *Hum Mol Genet*. 10:2821-31.

Matsushita, M., Y. Hosoya, and M. Ikeda. 1979. Anatomical organization of the spinocerebellar system in the cat, as studied by retrograde transport of horseradish peroxidase. *J Comp Neurol*. 184:81-106.

Maucuer, A., J.H. Camonis, and A. Sobel. 1995. Stathmin interaction with a putative kinase and coiled-coil-forming protein domains. *Proc Natl Acad Sci U S A*. 92:3100-4.

McGuire, S.O., Z.D. Ling, J.W. Lipton, C.E. Sortwell, T.J. Collier, and P.M. Carvey. 2001. Tumor necrosis factor alpha is toxic to embryonic mesencephalic dopamine neurons. *Exp Neurol*. 169:219-30.

McKay, B.E., J.D. Engbers, W.H. Mehaffey, G.R. Gordon, M.L. Molineux, J.S. Bains, and R.W. Turner. 2007. Climbing fiber discharge regulates cerebellar functions by controlling the intrinsic characteristics of purkinje cell output. *J Neurophysiol*. 97:2590-604.

McLean, G.W., N.H. Komiyama, B. Serrels, H. Asano, L. Reynolds, F. Conti, K. Hodivala-Dilke, D. Metzger, P. Chambon, S.G. Grant, and M.C. Frame.

2004. Specific deletion of focal adhesion kinase suppresses tumor formation and blocks malignant progression. *Genes Dev*. 18:2998-3003.

McNaught, K.S., R. Belizaire, O. Isacson, P. Jenner, and C.W. Olanow. 2003. Altered proteasomal function in sporadic Parkinson's disease. *Exp Neurol*. 179:38-46.

McNaught, K.S., R. Belizaire, P. Jenner, C.W. Olanow, and O. Isacson. 2002a. Selective loss of 20S proteasome alpha-subunits in the substantia nigra pars compacta in Parkinson's disease. *Neurosci Lett*. 326:155-8.

McNaught, K.S., and P. Jenner. 2001. Proteasomal function is impaired in substantia nigra in Parkinson's disease. *Neurosci Lett*. 297:191-4.

McNaught, K.S., C. Mytilineou, R. Jnobaptiste, J. Yabut, P. Shashidharan, P. Jennert, and C.W. Olanow. 2002b. Impairment of the ubiquitin-proteasome system causes dopaminergic cell death and inclusion body formation in ventral mesencephalic cultures. *J Neurochem*. 81:301-6.

Mehlhorn, G., M. Hollborn, and R. Schliebs. 2000. Induction of cytokines in glial cells surrounding cortical beta-amyloid plaques in transgenic Tg2576 mice with Alzheimer pathology. *Int J Dev Neurosci*. 18:423-31.

Melkoumian, Z.K., X. Peng, B. Gan, X. Wu, and J.L. Guan. 2005. Mechanism of cell cycle regulation by FIP200 in human breast cancer cells. *Cancer Res*. 65:6676-84.

Merrill, J.E. 1991. Effects of interleukin-1 and tumor necrosis factor-alpha on astrocytes, microglia, oligodendrocytes, and glial precursors in vitro. *Dev Neurosci*. 13:130-7.

Miale, I.L., and R.L. Sidman. 1961. An autoradiographic analysis of histogenesis in the mouse cerebellum. *Exp Neurol*. 4:277-96.

Micheau, O., and J. Tschopp. 2003. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell*. 114:181-90.

Millonig, J.H., K.J. Millen, and M.E. Hatten. 2000. The mouse Dreher gene Lmx1a controls formation of the roof plate in the vertebrate CNS. *Nature*. 403:764-9.

Mitra, S.K., D.A. Hanson, and D.D. Schlaepfer. 2005. Focal adhesion kinase: in command and control of cell motility. *Nat Rev Mol Cell Biol*. 6:56-68.

Mitra, S.K., and D.D. Schlaepfer. 2006. Integrin-regulated FAK-Src signaling in normal and cancer cells. *Curr Opin Cell Biol*. 18:516-23.

Mitsumoto, A., and Y. Nakagawa. 2001. DJ-1 is an indicator for endogenous reactive oxygen species elicited by endotoxin. *Free Radic Res*. 35:885-93.

Mizuno, Y., N. Hattori, H. Mori, T. Suzuki, and K. Tanaka. 2001. Parkin and Parkinson's disease. *Curr Opin Neurol*. 14:477-82.

Mizushima, N., B. Levine, A.M. Cuervo, and D.J. Klionsky. 2008. Autophagy fights disease through cellular self-digestion. *Nature*. 451:1069-75.

Mogi, M., M. Harada, P. Riederer, H. Narabayashi, K. Fujita, and T. Nagatsu. 1994. Tumor necrosis factor-alpha (TNF-alpha) increases both in the brain and in the cerebrospinal fluid from parkinsonian patients. *Neurosci Lett*. 165:208-10.

Mogi, M., A. Togari, T. Kondo, Y. Mizuno, O. Komure, S. Kuno, H. Ichinose, and T. Nagatsu. 2000. Caspase activities and tumor necrosis factor receptor R1 (p55) level are elevated in the substantia nigra from parkinsonian brain. *J Neural Transm*. 107:335-41.

Morganti-Kossman, M.C., P.M. Lenzlinger, V. Hans, P. Stahel, E. Csuka, E. Ammann, R. Stocker, O. Trentz, and T. Kossmann. 1997. Production of

cytokines following brain injury: beneficial and deleterious for the damaged tissue. *Mol Psychiatry*. 2:133-6.

Mugnaini, E., and A. Floris. 1994. The unipolar brush cell: a neglected neuron of the mammalian cerebellar cortex. *J Comp Neurol*. 339:174-80.

Munch, G., J. Apelt, E. Rosemarie Kientsch, P. Stahl, H.J. Luth, and R. Schliebs. 2003. Advanced glycation endproducts and pro-inflammatory cytokines in transgenic Tg2576 mice with amyloid plaque pathology. *J Neurochem*. 86:283-9.

Muratani, M., and W.P. Tansey. 2003. How the ubiquitin-proteasome system controls transcription. *Nat Rev Mol Cell Biol*. 4:192-201.

Nagase, T., N. Seki, K. Ishikawa, M. Ohira, Y. Kawarabayasi, O. Ohara, A. Tanaka, H. Kotani, N. Miyajima, and N. Nomura. 1996. Prediction of the coding sequences of unidentified human genes. VI. The coding sequences of 80 new genes (KIAA0201-KIAA0280) deduced by analysis of cDNA clones from cell line KG-1 and brain. *DNA Res*. 3:321-9, 341-54.

Nagatsu, T., M. Mogi, H. Ichinose, and A. Togari. 2000. Changes in cytokines and neurotrophins in Parkinson's disease. *J Neural Transm Suppl*:277-90.

Nakamura, K., S.Y. Jeong, T. Uchihara, M. Anno, K. Nagashima, T. Nagashima, S. Ikeda, S. Tsuji, and I. Kanazawa. 2001. SCA17, a novel autosomal dominant cerebellar ataxia caused by an expanded polyglutamine in TATA-binding protein. *Hum Mol Genet*. 10:1441-8.

Nishina, H., C. Vaz, P. Billia, M. Nghiem, T. Sasaki, J.L. De la Pompa, K. Furlonger, C. Paige, C. Hui, K.D. Fischer, H. Kishimoto, T. Iwatsubo, T. Katada, J.R. Woodgett, and J.M. Penninger. 1999. Defective liver formation and liver cell apoptosis in mice lacking the stress signaling kinase SEK1/MKK4. *Development*. 126:505-16.

Nishitoh, H., M. Saitoh, Y. Mochida, K. Takeda, H. Nakano, M. Rothe, K. Miyazono, and H. Ichijo. 1998. ASK1 is essential for JNK/SAPK activation by TRAF2. *Mol Cell*. 2:389-95.

Niwa, J., S. Ishigaki, N. Hishikawa, M. Yamamoto, M. Doyu, S. Murata, K. Tanaka, N. Taniguchi, and G. Sobue. 2002. Dorfin ubiquitylates mutant SOD1 and prevents mutant SOD1-mediated neurotoxicity. *J Biol Chem*. 277:36793-8.

Nixon, R.A., J. Wegiel, A. Kumar, W.H. Yu, C. Peterhoff, A. Cataldo, and A.M. Cuervo. 2005. Extensive involvement of autophagy in Alzheimer disease: an immuno-electron microscopy study. *J Neuropathol Exp Neurol*. 64:113-22.

Nussbaum, R.L., and M.H. Polymeropoulos. 1997. Genetics of Parkinson's disease. *Hum Mol Genet*. 6:1687-91.

Oberdick, J., K. Schilling, R.J. Smeyne, J.G. Corbin, C. Bocchiaro, and J.I. Morgan. 1993. Control of segment-like patterns of gene expression in the mouse cerebellum. *Neuron*. 10:1007-18.

Owens, L.V., L. Xu, R.J. Craven, G.A. Dent, T.M. Weiner, L. Kornberg, E.T. Liu, and W.G. Cance. 1995. Overexpression of the focal adhesion kinase (p125FAK) in invasive human tumors. *Cancer Res*. 55:2752-5.

Paganelli, R., A. Di Iorio, L. Patricelli, F. Ripani, E. Sparvieri, R. Faricelli, C. Iarlori, E. Porreca, M. Di Gioacchino, and G. Abate. 2002. Proinflammatory cytokines in sera of elderly patients with dementia: levels in vascular injury are higher than those of mild-moderate Alzheimer's disease patients. *Exp Gerontol*. 37:257-63.

Pallanck, L., and J.T. Greenamyre. 2006. Neurodegenerative disease: pink, parkin and the brain. *Nature*. 441:1058.

Park, J., S.B. Lee, S. Lee, Y. Kim, S. Song, S. Kim, E. Bae, J. Kim, M. Shong, J.M. Kim, and J. Chung. 2006. Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. *Nature*. 441:1157-61.

Perutz, M.F., and A.H. Windle. 2001. Cause of neural death in neurodegenerative diseases attributable to expansion of glutamine repeats. *Nature*. 412:143-4.

Petersen, A., K.E. Larsen, G.G. Behr, N. Romero, S. Przedborski, P. Brundin, and D. Sulzer. 2001. Expanded CAG repeats in exon 1 of the Huntington's disease gene stimulate dopamine-mediated striatal neuron autophagy and degeneration. *Hum Mol Genet*. 10:1243-54.

Pfeuffer, T., W. Goebel, J. Laubinger, and M. Bachmann. 2000. LaXp180, a mammalian ActA-binding protein, identified with the yeast two-hybrid system, co-localizes …. *Cellular Microbiology*.

Piccardo, P., J. Safar, M. Ceroni, D.C. Gajdusek, and C.J. Gibbs, Jr. 1990. Immunohistochemical localization of prion protein in spongiform encephalopathies and normal brain tissue. *Neurology*. 40:518-22.

Pickart, C.M., and R.E. Cohen. 2004. Proteasomes and their kin: proteases in the machine age. *Nat Rev Mol Cell Biol*. 5:177-87.

Pickering, M., D. Cumiskey, and J.J. O'Connor. 2005. Actions of TNF-alpha on glutamatergic synaptic transmission in the central nervous system. *Exp Physiol*. 90:663-70.

Pollanen, M.S., D.W. Dickson, and C. Bergeron. 1993. Pathology and biology of the Lewy body. *J Neuropathol Exp Neurol*. 52:183-91.

Qin, Z.H., Y. Wang, K.B. Kegel, A. Kazantsev, B.L. Apostol, L.M. Thompson, J. Yoder, N. Aronin, and M. DiFiglia. 2003. Autophagy regulates the

processing of amino terminal huntingtin fragments. *Hum Mol Genet*. 12:3231-44.

Rahman, M.M., and G. McFadden. 2006. Modulation of tumor necrosis factor by microbial pathogens. *PLoS Pathog*. 2:e4.

Rakic, P. 1971. Neuron-glia relationship during granule cell migration in developing cerebellar cortex. A Golgi and electronmicroscopic study in Macacus Rhesus. *J Comp Neurol*. 141:283-312.

Rakic, P., and R.L. Sidman. 1973. Organization of cerebellar cortex secondary to deficit of granule cells in weaver mutant mice. *J Comp Neurol*. 152:133-61.

Ramirez, A., A. Heimbach, J. Grundemann, B. Stiller, D. Hampshire, L.P. Cid, I. Goebel, A.F. Mubaidin, A.L. Wriekat, J. Roeper, A. Al-Din, A.M. Hillmer, M. Karsak, B. Liss, C.G. Woods, M.I. Behrens, and C. Kubisch. 2006. Hereditary parkinsonism with dementia is caused by mutations in ATP13A2, encoding a lysosomal type 5 P-type ATPase. *Nat Genet*. 38:1184-91.

Ravikumar, B., R. Duden, and D.C. Rubinsztein. 2002. Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. *Hum Mol Genet*. 11:1107-17.

Ravikumar, B., C. Vacher, Z. Berger, J.E. Davies, S. Luo, L.G. Oroz, F. Scaravilli, D.F. Easton, R. Duden, C.J. O'Kane, and D.C. Rubinsztein. 2004. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nat Genet*. 36:585-95.

Reggiori, F., and D.J. Klionsky. 2005. Autophagosomes: biogenesis from scratch? *Curr Opin Cell Biol*. 17:415-22.

Richly, H., M. Rape, S. Braun, S. Rumpf, C. Hoege, and S. Jentsch. 2005. A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. *Cell*. 120:73-84.

Rideout, H.J., I. Lang-Rollin, and L. Stefanis. 2004. Involvement of macroautophagy in the dissolution of neuronal inclusions. *Int J Biochem Cell Biol*. 36:2551-62.

Ross, C.A. 1997. Intranuclear neuronal inclusions: a common pathogenic mechanism for glutamine-repeat neurodegenerative diseases? *Neuron*. 19:1147-50.

Ross, C.A. 2002. Polyglutamine pathogenesis: emergence of unifying mechanisms for Huntington's disease and related disorders. *Neuron*. 35:819-22.

Rudolph, D., W.C. Yeh, A. Wakeham, B. Rudolph, D. Nallainathan, J. Potter, A.J. Elia, and T.W. Mak. 2000. Severe liver degeneration and lack of NF-kappaB activation in NEMO/IKKgamma-deficient mice. *Genes Dev*. 14:854-62.

Ruocco, M.G., S. Maeda, J.M. Park, T. Lawrence, L.C. Hsu, Y. Cao, G. Schett, E.F. Wagner, and M. Karin. 2005. I{kappa}B kinase (IKK){beta}, but not IKK{alpha}, is a critical mediator of osteoclast survival and is required for inflammation-induced bone loss. *J Exp Med*. 201:1677-87.

Sairanen, T., O. Carpen, M.L. Karjalainen-Lindsberg, A. Paetau, U. Turpeinen, M. Kaste, and P.J. Lindsberg. 2001. Evolution of cerebral tumor necrosis factor-alpha production during human ischemic stroke. *Stroke*. 32:1750-8.

Sakata, E., Y. Yamaguchi, E. Kurimoto, J. Kikuchi, S. Yokoyama, S. Yamada, H. Kawahara, H. Yokosawa, N. Hattori, Y. Mizuno, K. Tanaka, and K. Kato.

2003. Parkin binds the Rpn10 subunit of 26S proteasomes through its ubiquitin-like domain. *EMBO Rep*. 4:301-6.

Sato, S., H. Sanjo, K. Takeda, J. Ninomiya-Tsuji, M. Yamamoto, T. Kawai, K. Matsumoto, O. Takeuchi, and S. Akira. 2005. Essential function for the kinase TAK1 in innate and adaptive immune responses. *Nat Immunol*. 6:1087-95.

Scheper, W., and E.M. Hol. 2005. Protein quality control in Alzheimer's disease: a fatal saviour. *Curr Drug Targets CNS Neurol Disord*. 4:283-92.

Scherzinger, E., R. Lurz, M. Turmaine, L. Mangiarini, B. Hollenbach, R. Hasenbank, G.P. Bates, S.W. Davies, H. Lehrach, and E.E. Wanker. 1997. Huntingtin-encoded polyglutamine expansions form amyloid-like protein aggregates in vitro and in vivo. *Cell*. 90:549-58.

Schmelzle, T., and M.N. Hall. 2000. TOR, a central controller of cell growth. *Cell*. 103:253-62.

Schmidt, T., K.S. Lindenberg, A. Krebs, L. Schols, F. Laccone, J. Herms, M. Rechsteiner, O. Riess, and G.B. Landwehrmeyer. 2002. Protein surveillance machinery in brains with spinocerebellar ataxia type 3: redistribution and differential recruitment of 26S proteasome subunits and chaperones to neuronal intranuclear inclusions. *Ann Neurol*. 51:302-10.

Schneider-Brachert, W., V. Tchikov, J. Neumeyer, M. Jakob, S. Winoto-Morbach, J. Held-Feindt, M. Heinrich, O. Merkel, M. Ehrenschwender, D. Adam, R. Mentlein, D. Kabelitz, and S. Schutze. 2004. Compartmentalization of TNF receptor 1 signaling: internalized TNF receptosomes as death signaling vesicles. *Immunity*. 21:415-28.

Schwarz, C., and Y. Schmitz. 1997. Projection from the cerebellar lateral nucleus to precerebellar nuclei in the mossy fiber pathway is glutamatergic: a study combining anterograde tracing with immunogold labeling in the rat. *J Comp Neurol*. 381:320-34.

Sedgwick, J.D., D.S. Riminton, J.G. Cyster, and H. Korner. 2000. Tumor necrosis factor: a master-regulator of leukocyte movement. *Immunol Today*. 21:110-3.

Seil, F.J., M.L. Johnson, and R. Hawkes. 1995. Molecular compartmentation expressed in cerebellar cultures in the absence of neuronal activity and neuron-glia interactions. *J Comp Neurol*. 356:398-407.

Selmaj, K.W., M. Farooq, W.T. Norton, C.S. Raine, and C.F. Brosnan. 1990. Proliferation of astrocytes in vitro in response to cytokines. A primary role for tumor necrosis factor. *J Immunol*. 144:129-35.

Sheldon, M., D.S. Rice, G. D'Arcangelo, H. Yoneshima, K. Nakajima, K. Mikoshiba, B.W. Howell, J.A. Cooper, D. Goldowitz, and T. Curran. 1997. Scrambler and yotari disrupt the disabled gene and produce a reeler-like phenotype in mice. *Nature*. 389:730-3.

Sherman, M.Y., and A.L. Goldberg. 2001. Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. *Neuron*. 29:15-32.

Shibata, M., T. Lu, T. Furuya, A. Degterev, N. Mizushima, T. Yoshimori, M. MacDonald, B. Yankner, and J. Yuan. 2006. Regulation of intracellular accumulation of mutant Huntingtin by Beclin 1. *J Biol Chem*. 281:14474-85.

Shimura, H., M.G. Schlossmacher, N. Hattori, M.P. Frosch, A. Trockenbacher, R. Schneider, Y. Mizuno, K.S. Kosik, and D.J. Selkoe. 2001.

Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease. *Science*. 293:263-9.

Shinder, G.A., M.C. Lacourse, S. Minotti, and H.D. Durham. 2001. Mutant Cu/Zn-superoxide dismutase proteins have altered solubility and interact with heat shock/stress proteins in models of amyotrophic lateral sclerosis. *J Biol Chem*. 276:12791-6.

Shojaeian, H., N. Delhaye-Bouchaud, and J. Mariani. 1988. Stability of inferior olivary neurons in rodents. I. Moderate cell loss in adult Purkinje cell degeneration mutant mouse. *Brain Res*. 466:211-8.

Sikorska, B., P.P. Liberski, P. Giraud, N. Kopp, and P. Brown. 2004. Autophagy is a part of ultrastructural synaptic pathology in Creutzfeldt-Jakob disease: a brain biopsy study. *Int J Biochem Cell Biol*. 36:2563-73.

Singleton, A.B., M. Farrer, J. Johnson, A. Singleton, S. Hague, J. Kachergus, M. Hulihan, T. Peuralinna, A. Dutra, R. Nussbaum, S. Lincoln, A. Crawley, M. Hanson, D. Maraganore, C. Adler, M.R. Cookson, M. Muenter, M. Baptista, D. Miller, J. Blancato, J. Hardy, and K. Gwinn-Hardy. 2003. alpha-Synuclein locus triplication causes Parkinson's disease. *Science*. 302:841.

Sly, L.M., R.F. Krzesicki, J.R. Brashler, A.E. Buhl, D.D. McKinley, D.B. Carter, and J.E. Chin. 2001. Endogenous brain cytokine mRNA and inflammatory responses to lipopolysaccharide are elevated in the Tg2576 transgenic mouse model of Alzheimer's disease. *Brain Res Bull*. 56:581-8.

Snyder, H., K. Mensah, C. Theisler, J. Lee, A. Matouschek, and B. Wolozin. 2003. Aggregated and monomeric alpha-synuclein bind to the S6'

proteasomal protein and inhibit proteasomal function. *J Biol Chem*. 278:11753-9.

Sotelo, C., D.E. Hillman, A.J. Zamora, and R. Llinas. 1975. Climbing fiber deafferentation: its action on Purkinje cell dendritic spines. *Brain Res*. 98:574-81.

Sriram, K., J.M. Matheson, S.A. Benkovic, D.B. Miller, M.I. Luster, and J.P. O'Callaghan. 2002. Mice deficient in TNF receptors are protected against dopaminergic neurotoxicity: implications for Parkinson's disease. *FASEB J*. 16:1474-6.

Staropoli, J.F., C. McDermott, C. Martinat, B. Schulman, E. Demireva, and A. Abeliovich. 2003. Parkin is a component of an SCF-like ubiquitin ligase complex and protects postmitotic neurons from kainate excitotoxicity. *Neuron*. 37:735-49.

Stefanis, L., K.E. Larsen, H.J. Rideout, D. Sulzer, and L.A. Greene. 2001. Expression of A53T mutant but not wild-type alpha-synuclein in PC12 cells induces alterations of the ubiquitin-dependent degradation system, loss of dopamine release, and autophagic cell death. *J Neurosci*. 21:9549-60.

Stellwagen, D., E.C. Beattie, J.Y. Seo, and R.C. Malenka. 2005. Differential regulation of AMPA receptor and GABA receptor trafficking by tumor necrosis factor-alpha. *J Neurosci*. 25:3219-28.

Stellwagen, D., and R.C. Malenka. 2006. Synaptic scaling mediated by glial TNF-alpha. *Nature*. 440:1054-9.

Stenoien, D.L., C.J. Cummings, H.P. Adams, M.G. Mancini, K. Patel, G.N. DeMartino, M. Marcelli, N.L. Weigel, and M.A. Mancini. 1999. Polyglutamine-expanded androgen receptors form aggregates that

sequester heat shock proteins, proteasome components and SRC-1, and are suppressed by the HDJ-2 chaperone. *Hum Mol Genet*. 8:731-41.

Stenoien, D.L., M. Mielke, and M.A. Mancini. 2002. Intranuclear ataxin1 inclusions contain both fast- and slow-exchanging components. *Nat Cell Biol*. 4:806-10.

Sturrock, R.R. 1989. Age related changes in Purkinje cell number in the cerebellar nodulus of the mouse. *J Hirnforsch*. 30:757-60.

Tamura, M., J. Gu, K. Matsumoto, S. Aota, R. Parsons, and K.M. Yamada. 1998. Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. *Science*. 280:1614-7.

Tartaglia, L.A., T.M. Ayres, G.H. Wong, and D.V. Goeddel. 1993. A novel domain within the 55 kd TNF receptor signals cell death. *Cell*. 74:845-53.

Taylor, J.P., J. Hardy, and K.H. Fischbeck. 2002. Toxic proteins in neurodegenerative disease. *Science*. 296:1991-5.

Tobiume, K., A. Matsuzawa, T. Takahashi, H. Nishitoh, K. Morita, K. Takeda, O. Minowa, K. Miyazono, T. Noda, and H. Ichijo. 2001. ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. *EMBO Rep*. 2:222-8.

Tolbert, D.L., H. Bantli, and J.R. Bloedel. 1978. Organizational features of the cat and monkey cerebellar nucleocortical projection. *J Comp Neurol*. 182:39-56.

Tomoda, T., J.H. Kim, C. Zhan, and M.E. Hatten. 2004. Role of Unc51.1 and its binding partners in CNS axon outgrowth. *Genes Dev*. 18:541-58.

Triarhou, L.C., and B. Ghetti. 1991. Stabilisation of neurone number in the inferior olivary complex of aged 'Purkinje cell degeneration' mutant mice. *Acta Neuropathol*. 81:597-602.

Triarhou, L.C., J. Norton, and B. Ghetti. 1987. Anterograde transsynaptic degeneration in the deep cerebellar nuclei of Purkinje cell degeneration (pcd) mutant mice. *Exp Brain Res*. 66:577-88.

Tsai, Y.C., P.S. Fishman, N.V. Thakor, and G.A. Oyler. 2003. Parkin facilitates the elimination of expanded polyglutamine proteins and leads to preservation of proteasome function. *J Biol Chem*. 278:22044-55.

Ueda, H., S. Abbi, C. Zheng, and J.L. Guan. 2000. Suppression of Pyk2 kinase and cellular activities by FIP200. *J Cell Biol*. 149:423-30.

Upadhya, S.C., and A.N. Hegde. 2003. A potential proteasome-interacting motif within the ubiquitin-like domain of parkin and other proteins. *Trends Biochem Sci*. 28:280-3.

Urushitani, M., J. Kurisu, K. Tsukita, and R. Takahashi. 2002. Proteasomal inhibition by misfolded mutant superoxide dismutase 1 induces selective motor neuron death in familial amyotrophic lateral sclerosis. *J Neurochem*. 83:1030-42.

Valente, E.M., P.M. Abou-Sleiman, V. Caputo, M.M. Muqit, K. Harvey, S. Gispert, Z. Ali, D. Del Turco, A.R. Bentivoglio, D.G. Healy, A. Albanese, R. Nussbaum, R. Gonzalez-Maldonado, T. Deller, S. Salvi, P. Cortelli, W.P. Gilks, D.S. Latchman, R.J. Harvey, B. Dallapiccola, G. Auburger, and N.W. Wood. 2004. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science*. 304:1158-60.

Valentine, J.S., and P.J. Hart. 2003. Misfolded CuZnSOD and amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A*. 100:3617-22.

Varshavsky, A. 2005. Regulated protein degradation. *Trends Biochem Sci*. 30:283-6.

Verhoef, L.G., K. Lindsten, M.G. Masucci, and N.P. Dantuma. 2002. Aggregate formation inhibits proteasomal degradation of polyglutamine proteins. *Hum Mol Genet*. 11:2689-700.

Voigtlander, T., S. Kloppel, P. Birner, C. Jarius, H. Flicker, S. Verghese-Nikolakaki, T. Sklaviadis, M. Guentchev, and H. Budka. 2001. Marked increase of neuronal prion protein immunoreactivity in Alzheimer's disease and human prion diseases. *Acta Neuropathol*. 101:417-23.

Wada, T., N. Joza, H.Y. Cheng, T. Sasaki, I. Kozieradzki, K. Bachmaier, T. Katada, M. Schreiber, E.F. Wagner, H. Nishina, and J.M. Penninger. 2004. MKK7 couples stress signalling to G2/M cell-cycle progression and cellular senescence. *Nat Cell Biol*. 6:215-26.

Waelter, S., A. Boeddrich, R. Lurz, E. Scherzinger, G. Lueder, H. Lehrach, and E.E. Wanker. 2001. Accumulation of mutant huntingtin fragments in aggresome-like inclusion bodies as a result of insufficient protein degradation. *Mol Biol Cell*. 12:1393-407.

Wallace, V.A. 1999. Purkinje-cell-derived Sonic hedgehog regulates granule neuron precursor cell proliferation in the developing mouse cerebellum. *Curr Biol*. 9:445-8.

Wang, V.Y., and H.Y. Zoghbi. 2001. Genetic regulation of cerebellar development. *Nat Rev Neurosci*. 2:484-91.

Wang, Y., R. Singh, J.H. Lefkowitch, R.M. Rigoli, and M.J. Czaja. 2006. Tumor necrosis factor-induced toxic liver injury results from JNK2-dependent activation of caspase-8 and the mitochondrial death pathway. *J Biol Chem*. 281:15258-67.

Ware, C.F. 2005. Network communications: lymphotoxins, LIGHT, and TNF. *Annu Rev Immunol*. 23:787-819.

Ware, C.F., S. VanArsdale, and T.L. VanArsdale. 1996. Apoptosis mediated by the TNF-related cytokine and receptor families. *J Cell Biochem*. 60:47-55.

Wassef, M., J. Simons, M.L. Tappaz, and C. Sotelo. 1986. Non-Purkinje cell GABAergic innervation of the deep cerebellar nuclei: a quantitative immunocytochemical study in C57BL and in Purkinje cell degeneration mutant mice. *Brain Res*. 399:125-35.

Wassef, M., C. Sotelo, M. Thomasset, A.C. Granholm, N. Leclerc, J. Rafrafi, and R. Hawkes. 1990. Expression of compartmentation antigen zebrin I in cerebellar transplants. *J Comp Neurol*. 294:223-34.

Watanabe, R., T. Chano, H. Inoue, T. Isono, O. Koiwai, and H. Okabe. 2005. Rb1cc1 is critical for myoblast differentiation through Rb1 regulation. *Virchows Arch*. 447:643-8.

Webb, J.L., B. Ravikumar, J. Atkins, J.N. Skepper, and D.C. Rubinsztein. 2003. Alpha-Synuclein is degraded by both autophagy and the proteasome. *J Biol Chem*. 278:25009-13.

Wei, H., B. Gan, X. Wu, and J.L. Guan. 2009. Inactivation of FIP200 Leads to Inflammatory Skin Disorder, but Not Tumorigenesis, in Conditional Knock-out Mouse Models. *J Biol Chem*. 284:6004-13.

West, A., M. Periquet, S. Lincoln, C.B. Lucking, D. Nicholl, V. Bonifati, N. Rawal, T. Gasser, E. Lohmann, J.F. Deleuze, D. Maraganore, A. Levey, N. Wood, A. Durr, J. Hardy, A. Brice, and M. Farrer. 2002. Complex relationship between Parkin mutations and Parkinson disease. *Am J Med Genet*. 114:584-91.

Wullschleger, S., R. Loewith, and M.N. Hall. 2006. TOR signaling in growth and metabolism. *Cell*. 124:471-84.

Xiong, W., and J.T. Parsons. 1997. Induction of apoptosis after expression of PYK2, a tyrosine kinase structurally related to focal adhesion kinase. *J Cell Biol*. 139:529-39.

Xue, L., G.C. Fletcher, and A.M. Tolkovsky. 1999. Autophagy is activated by apoptotic signalling in sympathetic neurons: an alternative mechanism of death execution. *Mol Cell Neurosci*. 14:180-98.

Yedidia, Y., L. Horonchik, S. Tzaban, A. Yanai, and A. Taraboulos. 2001. Proteasomes and ubiquitin are involved in the turnover of the wild-type prion protein. *EMBO J*. 20:5383-91.

Yi, J.J., and M.D. Ehlers. 2005. Ubiquitin and protein turnover in synapse function. *Neuron*. 47:629-32.

Yu, D.H., C.K. Qu, O. Henegariu, X. Lu, and G.S. Feng. 1998. Protein-tyrosine phosphatase Shp-2 regulates cell spreading, migration, and focal adhesion. *J Biol Chem*. 273:21125-31.

Yu, W.H., A.M. Cuervo, A. Kumar, C.M. Peterhoff, S.D. Schmidt, J.H. Lee, P.S. Mohan, M. Mercken, M.R. Farmery, L.O. Tjernberg, Y. Jiang, K. Duff, Y. Uchiyama, J. Naslund, P.M. Mathews, A.M. Cataldo, and R.A. Nixon. 2005. Macroautophagy--a novel Beta-amyloid peptide-generating pathway activated in Alzheimer's disease. *J Cell Biol*. 171:87-98.

Yuan, J., M. Lipinski, and A. Degterev. 2003. Diversity in the mechanisms of neuronal cell death. *Neuron*. 40:401-13.

Zanjani, H., Y. Lemaigre-Dubreuil, N.J. Tillakaratne, A. Blokhin, R.P. McMahon, A.J. Tobin, M.W. Vogel, and J. Mariani. 2004. Cerebellar Purkinje cell loss in aging Hu-Bcl-2 transgenic mice. *J Comp Neurol*. 475:481-92.

Zanjani, H.S., M.W. Vogel, N. Delhaye-Bouchaud, J.C. Martinou, and J. Mariani. 1996. Increased cerebellar Purkinje cell numbers in mice overexpressing a human bcl-2 transgene. *J Comp Neurol*. 374:332-41.

Zanusso, G., R.B. Petersen, T. Jin, Y. Jing, R. Kanoush, S. Ferrari, P. Gambetti, and N. Singh. 1999. Proteasomal degradation and N-terminal protease resistance of the codon 145 mutant prion protein. *J Biol Chem*. 274:23396-404.

Zhang, Y., J. Gao, K.K. Chung, H. Huang, V.L. Dawson, and T.M. Dawson. 2000. Parkin functions as an E2-dependent ubiquitin- protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. *Proc Natl Acad Sci U S A*. 97:13354-9.

Zhao, Y., S. Li, E.E. Childs, D.K. Kuharsky, and X.M. Yin. 2001. Activation of pro-death Bcl-2 family proteins and mitochondria apoptosis pathway in tumor necrosis factor-alpha-induced liver injury. *J Biol Chem*. 276:27432-40.

Zoghbi, H.Y., and H.T. Orr. 2000. Glutamine repeats and neurodegeneration. *Annu Rev Neurosci*. 23:217-47.

Zuccato, C., M. Tartari, A. Crotti, D. Goffredo, M. Valenza, L. Conti, T. Cataudella, B.R. Leavitt, M.R. Hayden, T. Timmusk, D. Rigamonti, and E. Cattaneo. 2003. Huntingtin interacts with REST/NRSF to modulate the transcription of NRSE-controlled neuronal genes. *Nat Genet*. 35:76-83.

Chapter 2
CHARACTERIZATION of CEREBELLAR DEGENERATION MEDIATED by ABLATION of FIP200

2.1 Introduction

The cerebellum provides central control of posture, balance, and coordination of movements. Cerebellar development initiates during embryogenesis but extends into early postnatal development to achieve the highly organized cerebellar cortex (Kandel et al., 2000). Consistent with their crucial roles in the control of movement coordination, damages to cerebellum caused by either injury or neurodegeneration are associated with various degrees of ataxia phenotype both in human as well as mouse models. Indeed, the dominantly inherited ataxias or spinocerebellar ataxias are one of the most common forms of neurodegenerative diseases caused by progressive degeneration of the cerebellum. Studies in the last decade have identified various gene mutations underlying the different neurodegenerative disorders and revealed a potentially common cellular mechanism of these diseases, i.e. the accumulation of abnormal protein aggregation based on converging lines of investigations (Ross and Poirier, 2004). Protein quality control mechanisms including the ubiquitin-proteasome system and autophagy have been shown to play crucial roles in the homeostasis of neurons and neurodegeneration (Giobbe et al., 1991; Winslow and Rubinsztein, 2008). However our understanding of the molecular and cellular mechanisms by which key signaling molecules and pathways regulate these cellular processes in neurodegeneration is still incomplete at present.

FIP200 (FAK-family interacting protein of 200 kDa) (also called RB1CC1) is a 200 kDa protein that is characterized by a large coiled-coil region (Chano

et al., 2002b; Ueda et al., 2000). It was initially identified as a protein that interacts with Pyk2 and FAK leading to the inhibition of their kinase activities (Ueda et al., 2000) but has been shown to associate with several other cellular proteins and regulate several signaling pathways (Gan and Guan, 2008). FIP200 is widely expressed in various human tissues (Bamba et al., 2004) and is an evolutionarily conserved protein present in human, mouse, rat, frog, fly and worm. The high degree of conservation during evolution suggests potentially important functions of FIP200 *in vivo*. This is supported by our recent studies showing that total KO of FIP200 in mice resulted in embryonic lethality at mid/late gestation associated with heart failure and liver degeneration (Gan et al., 2006). Furthermore, it was recently reported that RNAi-mediated knockdown of FIP200 caused neurite atrophy and apoptosis in a neuroblastoma cell line Neuro-2a (Chano et al., 2007), suggesting that FIP200 may also play a role in neurons.

To explore a potential role of FIP200 in neurodegeneration *in vivo*, neuron-specific conditional FIP200 KO mice were generated and analyzed using the Cre-loxP approach. I found that conditional KO of FIP200 by nestin-Cre resulted in severe neurological defects including cerebellar degeneration and ataxia, which were accompanied by progressive loss of the Purkinje cells, spongiform degeneration, abnormal accumulation of ubiquitinated protein aggregates, and increased apoptosis in the cerebellum.

2.2 Material and Methods

Animals and genotyping

FIP200$^{flox/flox}$ mice were described previously (Gan et al., 2006). Nestin-Cre transgenic mice (Tronche et al., 1999) were obtained from The Jackson

Laboratory (Bar Harbor, ME). Mice were housed and handled according to local, state, and federal regulations, and all experimental procedures were carried out according to the guidelines of Institutional Animal Care and Use Committee at Cornell University and the University of Michigan. Mice genotyping for FIP200 and Cre alleles were performed by PCR analysis of tail DNA, essentially as described previously (Gan et al., 2006; Wei et al., 2009).

Protein extraction, SDS-PAGE and Western Blotting

The mouse brain tissues were collected from control or *FIP200*$^{flox/flox}$; nestin-Cre mice at P0. The protein lysates were prepared by homogenization in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.2% sodium deoxycholate, 0.2% sodium dodecylsulfate (SDS), 1 mM sodium ethylenediaminetetraacetate) supplemented with protease inhibitors (5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Tissue and cell debris were removed by centrifugation and cleared supernatants were further transferred into a clean microcentrifuge tube. Protein concentration was determined using Bio-Rad protein assay reagent. The lysates were boiled for 5 min in 1 x SDS sample buffer (50 mM Tris-HCl pH 6.8, 12.5% glycerol, 1% SDS, 0.01% bromophenol blue) containing 5% β-mercaptoethanol and were then resolved with 6% polyacrylamide gel electrophoresis. After the SDS-PAGE were transferred onto a nitrocellulose membrane, membranes were incubated with antibody against FIP200 (1:1000) for 1 h at room temperature, washed using TBST, and incubated with the HRP anti-rabbit secondary antibodies (1:5000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). An Enhanced Chemiluminescent (ECL) kit (Pierce) was used to detect the immunoreactivity signal.

Histology and Immunohistochemistry

Mice were euthanized using CO_2, and a complete tissue set was harvested during necropsy. Fixation was carried out for 16 h at 4°C using freshly made, pre-chilled (4°C) PBS-buffered formalin. The brain tissues were all sagittal sectioned and then embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin for routine histological examination or left unstained for immunohistochemistry. Hematoxylin- and eosin-stained sections were examined under an Olympus BX41 light microscope (Olympus America, Inc., Center Valley, PA), and images were captured with an Olympus digital camera (model DP70) using a DP Controller software (Version 1.2.1.10 [EC] 8). For immunohistochemistry, unstained tissue sections were first deparaffinized in 3 washes of xylene (3 min each) and were rehydrated in graded ethanol solutions (100, 95, 70, 50, and 30%). After heat-activated antigen retrieval (model Retriever 2000, PickCellLaboratories B.V., Amsterdam, Holland) according to manufacturer's specifications, sections were treated with blocking solutions; first with Avidin-Biotin Block (Dako Corp., Carpinteria, CA) then with Protein Block Serum Free (Dako Corp.). Sections were then incubated with the primary antibody (calbindin 1:2000, Sigma-Aldrich; ubiquitin 1:500, Cell signaling; Dako Corp.; GFAP 1:1000, Dako Corp.; MBP 1:1000, Millipore) at 37 °C for 1 h in a humid chamber, washed in PBS 3 times (5 min each), then incubated with the biotinylated secondary antibody anti-mouse for calbindin, ubiquitin (Cell signaling); anti-rabbit for FIP200, GFAP, ubiquitin (Dako Corp.); anti-rat (Vector) for MBP (1:200 dilution, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in a humidified chamber for 1 h at 37 °C and washed in PBS similarly as before. Finally, sections were incubated with horseradish peroxidase-streptavidin (ABC Elite kit, Vector Laboratories) for 15

min at room temperature in a humid chamber and washed with PBS similarly as before. As the last staining step, 3,3'-diaminobenzidine (SIGMA FAST® DAB with Metal Enhancer, Sigma-Aldrich) was added to the sections and incubated at room temperature until a macroscopically appreciable light brown color developed inthe sections (generally 30 s to 5 min). After incubation with DAB, sections were lightly counterstained will Gill's hematoxylin. Histological examination and digital photography were carried out as described previously.

Transmission electron microscopy

Samples were fixed in 2.5% glutaraldehyde in 0.1 M Sorensen's buffer, pH 7.4, overnight at 4°C. After several rinses with buffer, they were postfixed in 1% osmium tetroxide in the same buffer. They were then rinsed in double distilled water to remove phosphate salt and then stained with aqueous 3% uranyl acetate for one hour. The samples were dehydrated in ascending concentrations of ethanol, rinsed two times in propolynoxide, and embedded in epoxy resin. They were ultra-thin sectioned at 70 nm in thickness and stained with uranyl acetate and lead citrate. The sections were examined using a Philips CM100 electron microscope at 60 kV. Images were recorded digitally using an Hamamatsu ORCA-HR digital camera system operated using AMT software (Advanced Microscopy Techniques Corp., Danvers, MA).

***In situ* detection of apoptosis**

Paraffin-embedded embryo sections (6 μm) were deparaffinized, incubated in methanol containing 0.3% H_2O_2 for 30 min, washed, and incubated with proteinase K (20 μg/ml) in PBS for 15 min at room temperature. Apoptotic

cells were detected as described in the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA). Sections were counterstained with methyl green.

Cerebellar primary culture

Postnatal pups were killed by decapitation at P0 age. Cerebella were dissected, freed of meninges, collected in calcium and magnesium-free phosphate-buffered saline (CMF-PBS, pH7.4). Cerebella were further digested with trypsin (1%; Invitrogen Corp.) in CMF-PBS for 3 min at room temperature. Tissue was then triturated sequentially with fire-polished Pasteur pipettes in CMF-PBS supplemented with 0.05% DNAase I (Invitrogen Corp.) and 12 mM $MgSO_4$, to yield a single-cell suspension. Cells were spun down at 3,000g for 5 min and resuspended in Neurobasal media (Invitrogen Corp.) supplemented with B-27, Glutamax-1 (Gibco Corp.), penicillin-streptomycin, and 10% horse serum. Cell were plated at a density of 11 x 10^5 cells per cm^2 on 12 mm diameter poly-L-lysine (20 μg/ml) precoated glass coverslips for 1 h at 37 °C with 100% humidity and 5% CO_2. After cells had attached (3-5 h), the medium was changed into Serum-Free Neurobasal medium. Cultures were maintained at 37 °C with 100% humidity and 5% CO_2 for another 7 days.

Immunofluorescence and Nuclear Staining

The cerebellar neuronal cells were grown on glass coverslips in 24-well plates for 7 days. After 7 days, the cells were fixed with 4% paraformaldehyde at room temperature for 20 min, and permeabilized for 15 min by PBS containing 0.3% Triton X-100. For calbindin staining, the cells were incubated with monoclonal anti-calbindin (1:200 dilution in PBS) for 1 h at room temperature.

After washed in PBS, the cells were incubated with fluorescein isothiocyanate-conjugated secondary antibody for 1 h. For nuclear staining, the cells were incubated with 0.5 mg/ml Hoechst 33258 (Sigma) for 10 min at room temperature. Preparations were then washed in PBS, mounted in anti-fading solution (20 mM n-propyl gallate in 80% glycerol), and analyzed by an immunofluorescent microscopy (Olympus).

2.3 Results

2.3.1 Ablation of FIP200 in the neuronal precursors leads to severe neurological defects in mice

To study potential role of FIP200 in the central nervous system, the floxed *FIP200* (*FIP200*$^{flox/flox}$) mice, in which exons 4 and 5 of FIP200 gene are flanked by two loxP sequences (Gan et al., 2006), were crossed with the nestin-Cre transgenic mice which express Cre recombinase in neural precursors from E10.5 (Bates et al., 1999; Dahlstrand et al., 1995; Frappart et al., 2007). Cre-mediated deletion of exons 4 and 5 leads to a frame-shift mutation because of direct splicing from exon 3 (containing ATG codon) to exon 6, producing a small truncated and nonfunctional peptide. In contrast to embryonic lethality of total KO of FIP200 (Gan et al., 2006), *FIP200*$^{flox/flox}$; nestin-Cre (designated as Nestin-CKO) mice were born at the expected Mendelian ratio. However, approximately 45% of the mutant mice died shortly after birth within the first few days. The remaining mice also showed a significantly reduced survival rate after P14 and all mutant mice died by 60 days (Figure 2.1). The mutant mice showed growth retardation as compared to the control littermates starting from P7. By P21, the average weight and length of the mutant mice reached only about 50% and 75%, respectively, of the control mice (Figure 2.2).

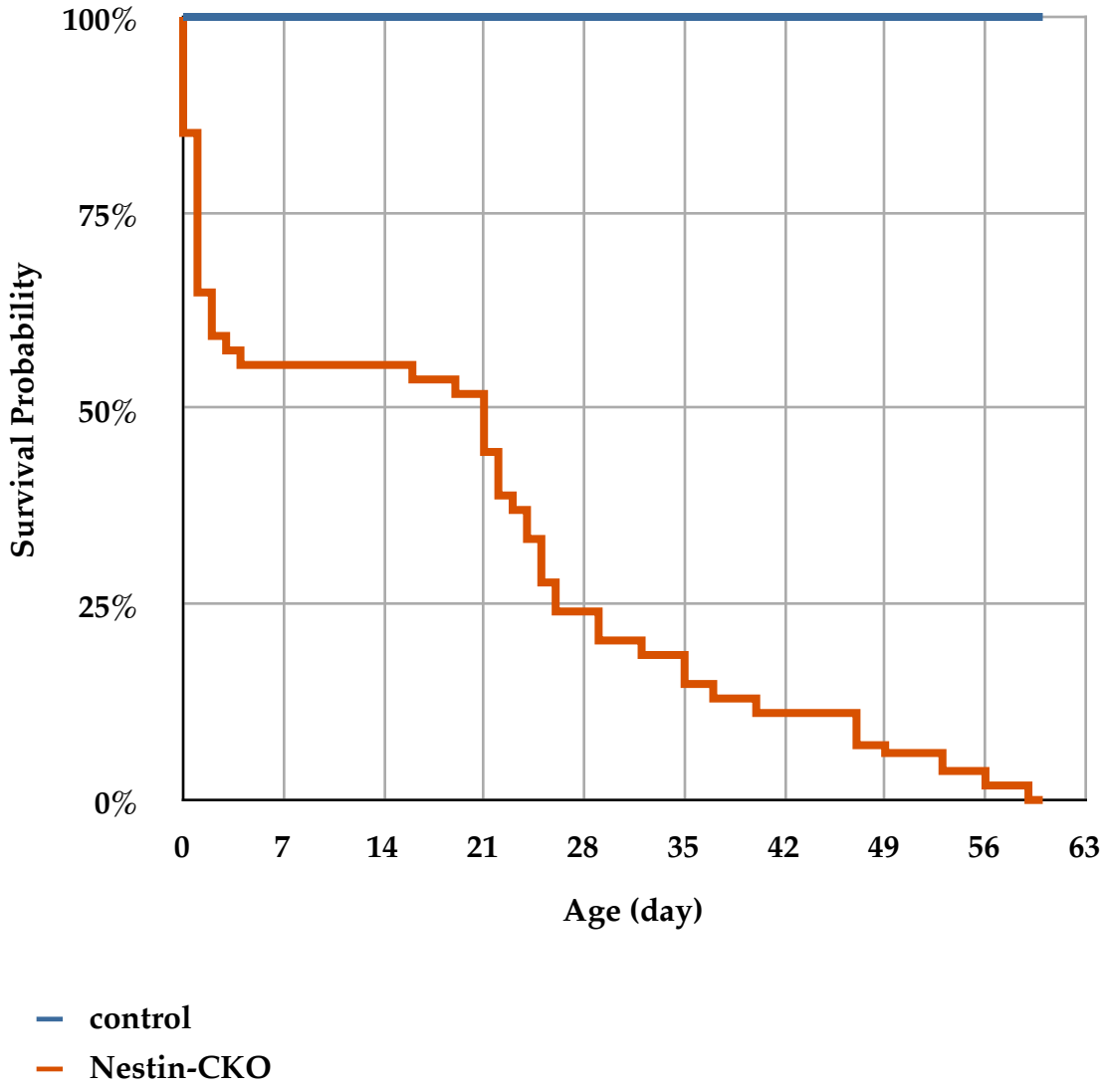


Figure 2.1 | Conditional ablation of FIP200 by nestin-Cre causes early death. Conditional ablation of FIP200 by nestin-Cre causes early death, growth retardation and cerebellar ataxia. Kaplan-Meier survival curve of control and Nestin-CKO mice (n=53).

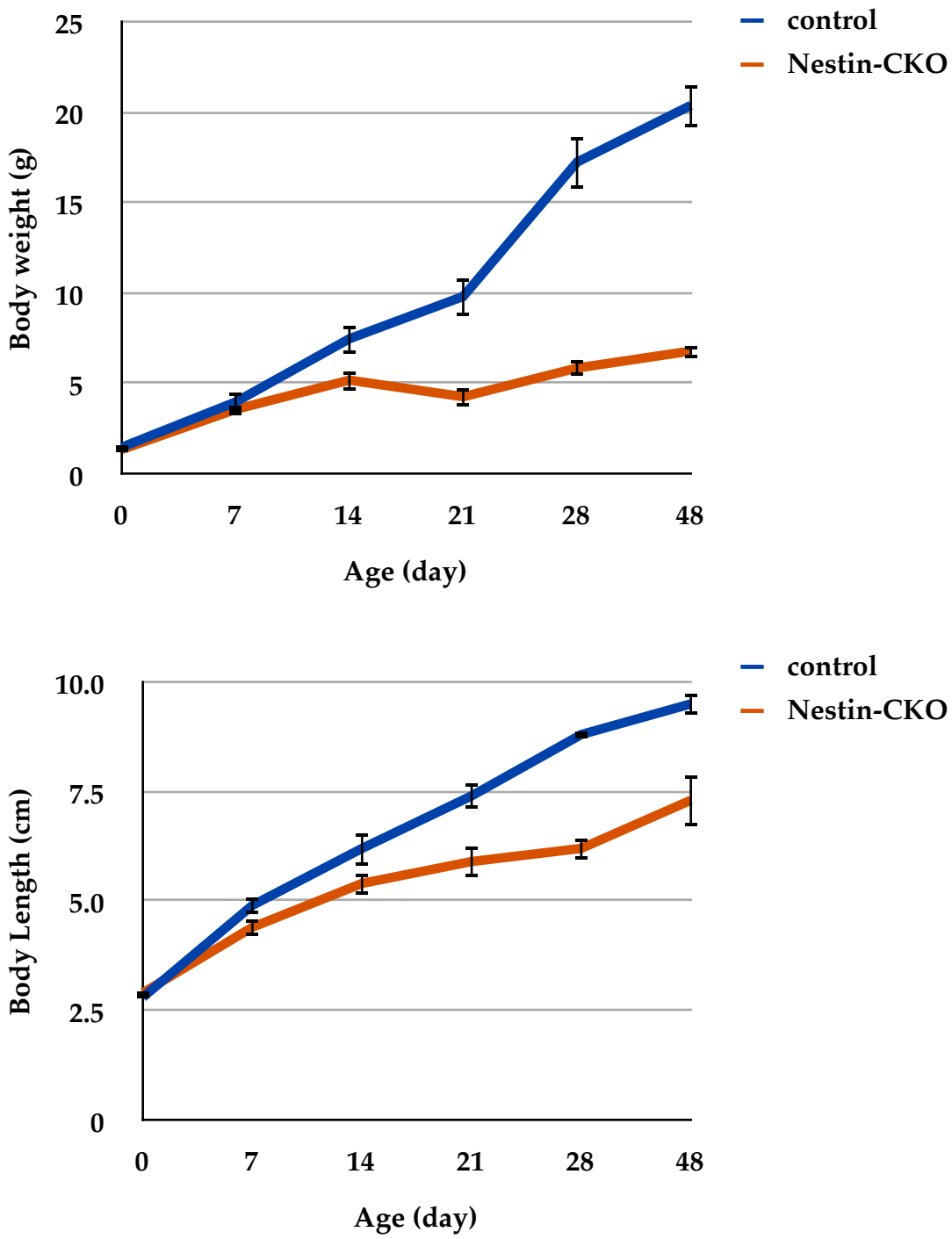


Figure 2.2 | Conditional ablation of FIP200 by nestin-Cre causes growth retardation. Body weight (top) and length (bottom) of control and Nestin-CKO mice at various days after birth.

Moreover, the mutant mice exhibited deficits in motor coordination starting around P14, which progressed to a severer extent with tremors, hyperactivity, and stiffness with movement. When they were suspended by their tails, the mutant mice showed the hind-limb crosses phenotype, whereas the control mice had the normally extended limbs (Figure 2.3). Consistent with the ataxia phenotype, histological analysis of the cerebella showed a smaller size for the mutant mice compared to the controls (Figure 2.4, panel A). The reduced cerebellum size is not a consequence of the smaller body size as the total size of the brain of the mutant mice was comparable to the controls (Figure 2.4, panel B). The foliation and fissuration appeared to be slightly less developed and the granule cell layer was thinner in the mutant cerebellum compared to the controls.

To evaluate the depletion of FIP200 in the mutant mice, lysates prepared from various regions of the brain of the mutant and control mice were subjected to Western blotting analysis. Figure 2.5 shows that FIP200 was deleted efficiently in several regions of the mutant brain including cerebral cortex, hypothalamus, midbrain, medulla and cerebellum. Because of the severe ataxia phenotype of the Nestin-CKO mice, I focused my analysis on cerebellum and further examined FIP200 expression by immunohistochemistry. As shown in Figure 2.6, FIP200 was eminently expressed in Purkinje cells, some cells in the granular layer, and deep cerebellar nuclei in the white matter of the control mice. In contrast, FIP200 was not detected in these cells of Nestin-CKO mice, confirming inactivation of FIP200 in the cerebella of the mutant mice. Together, these results suggest that deletion of FIP200 by nestin-Cre led to the development of cerebellar ataxia in mice.

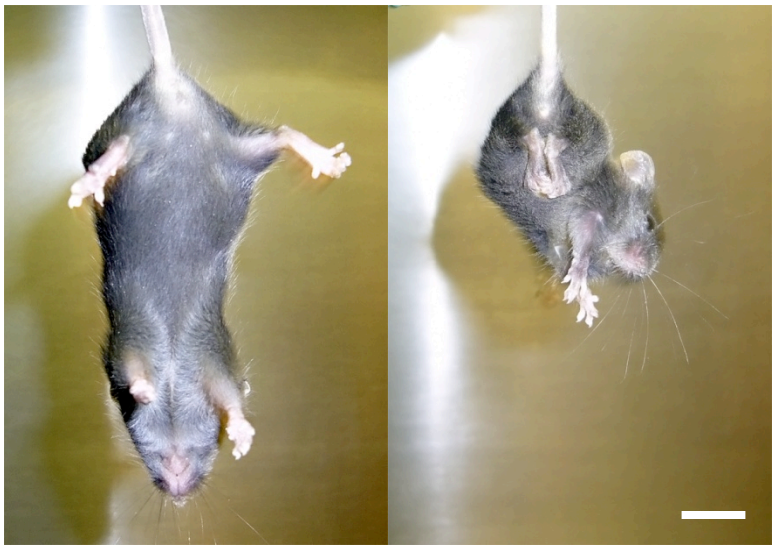


Figure 2.3 | Conditional ablation of FIP200 by nestin-Cre causes ataxia. Control mice (left) extend their hind limbs and bodies, whereas Nestin- CKO mice (right) show abnormal limb-clasping reflexes by crossing their limbs, when suspended by tail. Scale bar=1 cm.

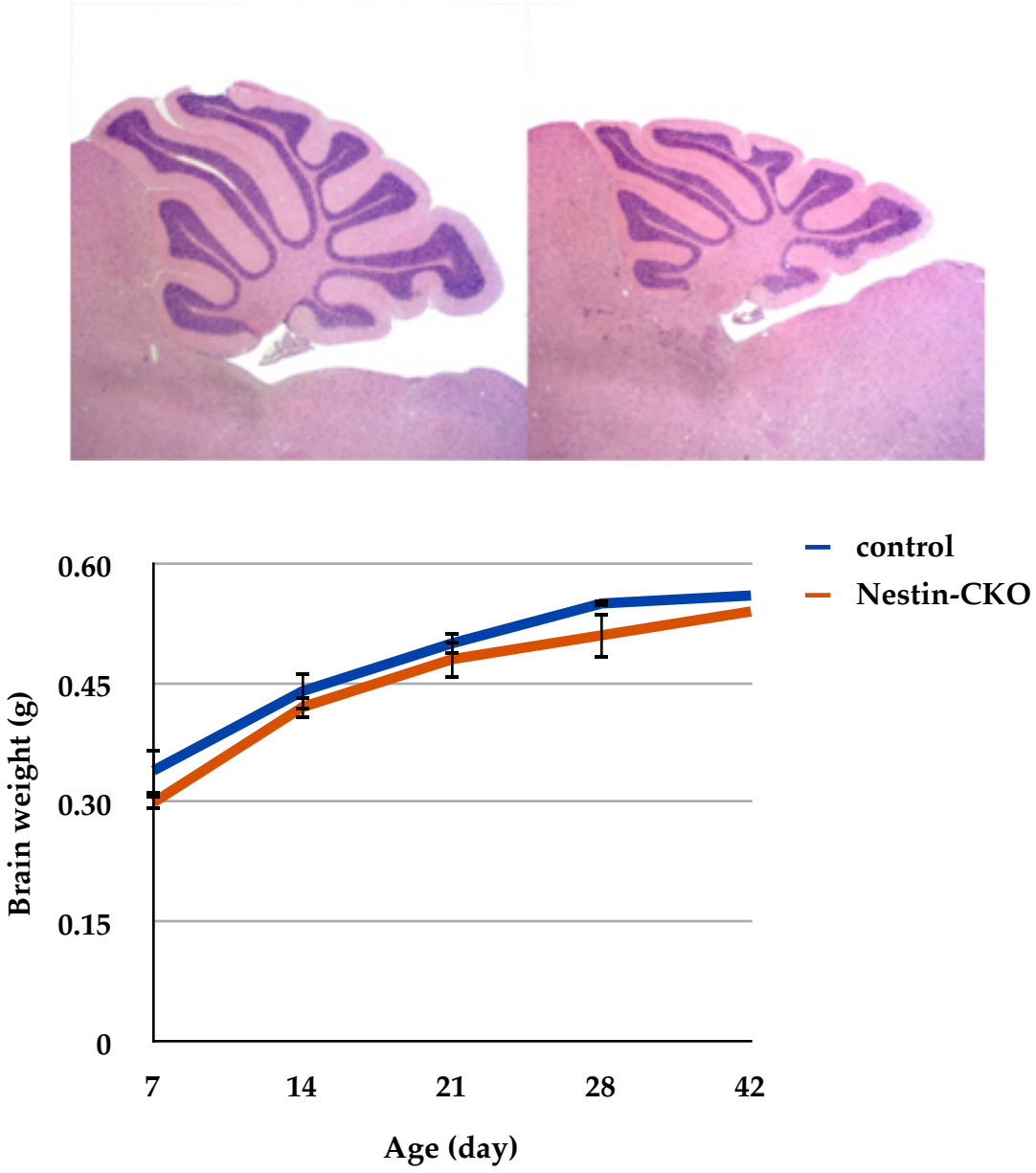


Figure 2.4 | Conditional ablation of FIP200 by nestin-Cre causes cerebellar degeneration. (A) Histological analysis of sagittal sections of cerebellum from control and Nestin-CKO mice at P28. Note the slightly smaller cerebellum and thinner granule cell layer in CKO mice. Scale bar=1 mm. (B) Brain weight of control and Nestin-CKO mice at various days after birth.

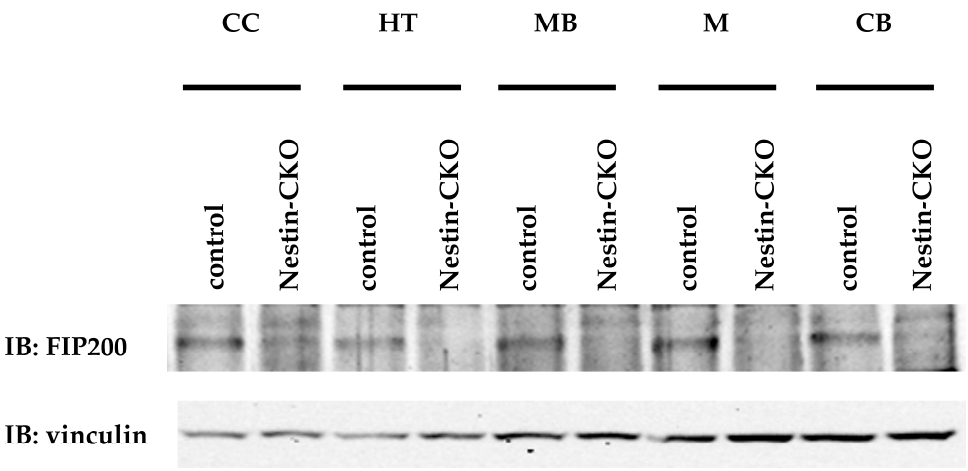


Figure 2.5 | Deletion of FIP200 in cerebellum analyzed by Western. Lysates were prepared from cerebral cortex (CC), hypothalamus (HT), midbrain (MB), medulla (M) and cerebellum (CB) of control or Nestin-CKO mice at P0 and then analyzed by Western blotting using anti-FIP200 (upper) or anti-vinculin (lower) antibodies.

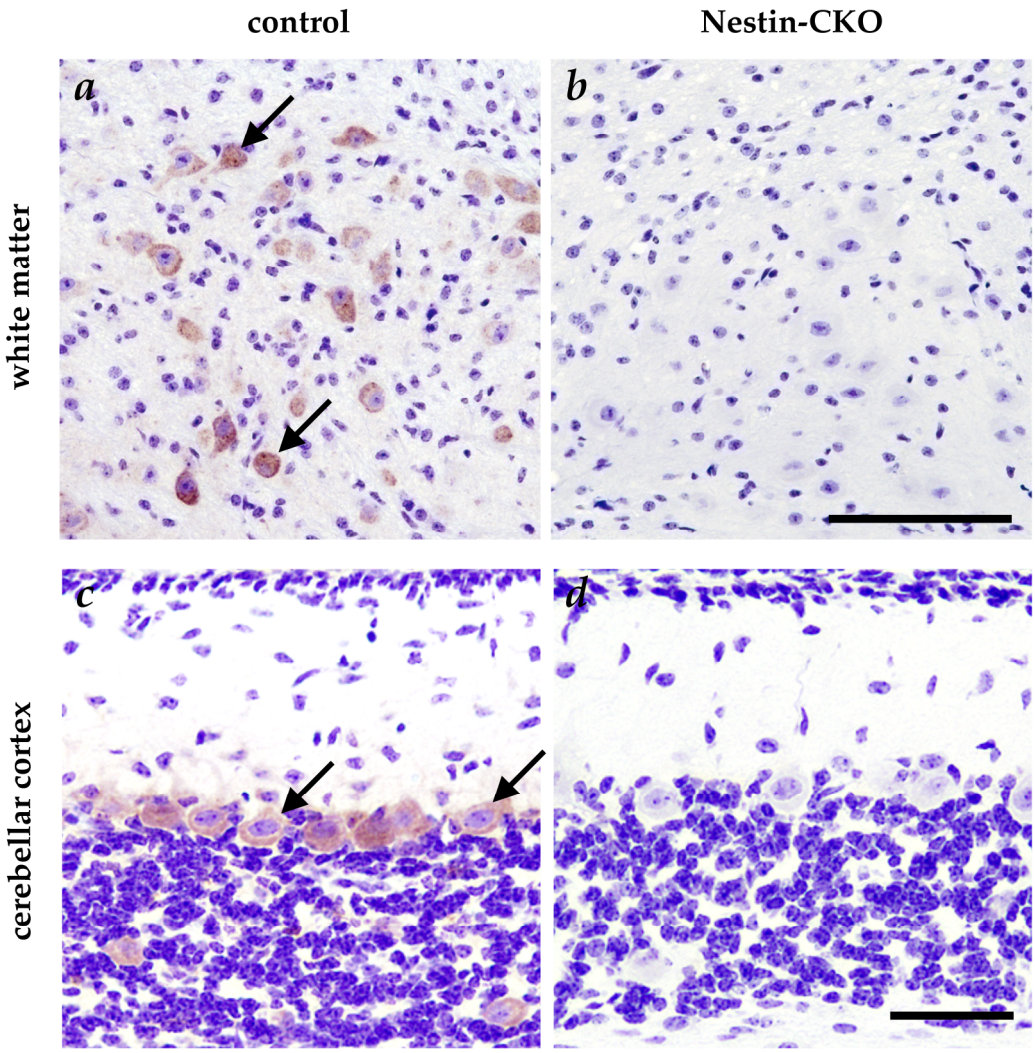


Figure 2.6 | Deletion of FIP200 in Nestin-CKO cerebellum analyzed by immunohistochemistry. Cerebella harvested from control (*a* and *c*) or Nestin-CKO (*b* and *d*) mice at P14 were sectioned and analyzed by immunohistochemistry with anti-FIP200. FIP200 is detected in the deep cell nuclei (*a*) and Purkinje cells (*c*) in the control (arrows), but not Nestin-CKO cerebellum (*b* and *d*). Scale bars=200 μm (*a* and *b*) and 100 μm (*c* and *d*), respectively.

2.3.2 Deletion of FIP200 results in progressive loss of Purkinje cells, spongiform and neurite degeneration in the cerebellum

To study the cerebellum defects in Nestin-CKO mice, histological analyses were performed on cerebella of the control and mutant mice at various stages of postnatal development. At P7 (data not shown) and P14 (Figure 2.7 panel *a* and *b*), the cellular organization of the cerebella was comparable between the Nestin-CKO and control mice although the external granular layer (EGL) appeared slightly thinner in the mutant mice. After P14, Nestin-CKO mice showed a progressive loss of Purkinje cells and the interneurons in the molecular layer as well as a gradual decrease in the thickness of the molecular layer relative to those in the control mice. Furthermore, signs of spongiform degeneration were observed in the white matter of the mutant mice at P14, which progressed to a very significant level at P56 (arrows, Figure 2.8 panel *f*). At P56, I also observed severe spongiform degeneration in the molecular layer and a slight decrease in the granule cells of the mutant cerebellum (Figure 2.7 panel *f*).

To further determine defects of Purkinje cells in the mutant mice, cerebellum sections were analyzed by immunohistochemistry using antibody against calbindin, a marker for Purkinje cells. Consistent with histological analysis, Figure 2.9 shows a decreased number of Purkinje cells in Nestin-CKO mice compared to the control mice (Figure 2.9 *a* and *b*). The staining also revealed extensive degeneration of dendrites in the mutant mice (Figure 2.9 compare panels *a* and *b*). Furthermore, calbindin labels were also found on particles that are slightly smaller than Purkinje cells in the granular layer (arrows), which could be segments of swelling (degenerating and/or abnormal) axons from the Purkinje cells traversing the granular layer.

Figure 2.7 | Deletion of FIP200 in cerebellum leads to progressive loss of Purkinje cells and spongiform degeneration in Nestin-CKO mice. Cerebellar sections from control (*a*, *c*, *e*) and Nestin-CKO (*b*, *d*, *f*) mice at different ages were stained by H&E. External granular layer (EGL), molecular layer (ML), Purkinje cell layer (PCL) and internal granular layer (GL) are marked on the left. Insets show high magnification view of bracketed areas in *e* and *f*. Arrows mark spongiform degeneration in the molecular layer. Scale bars=100 μm.

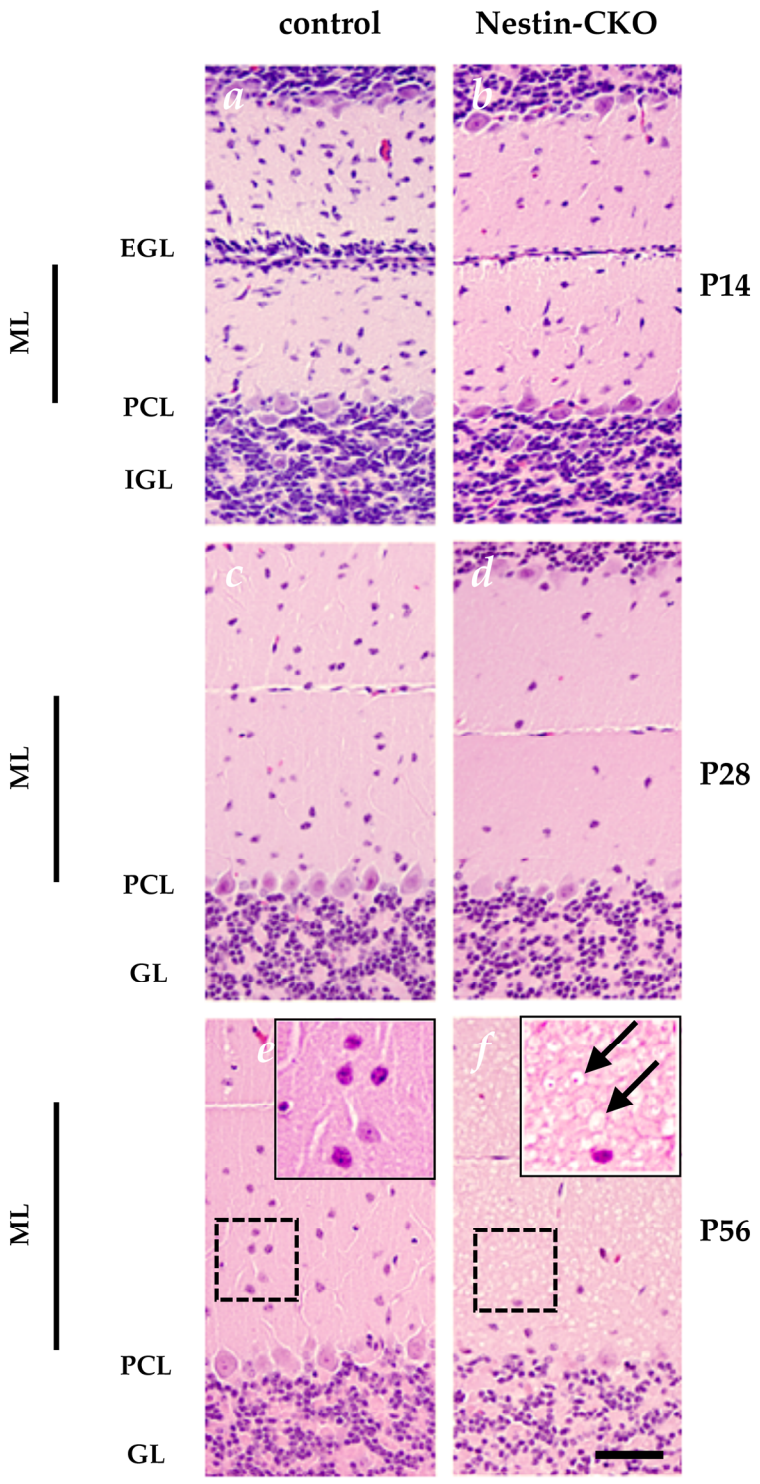
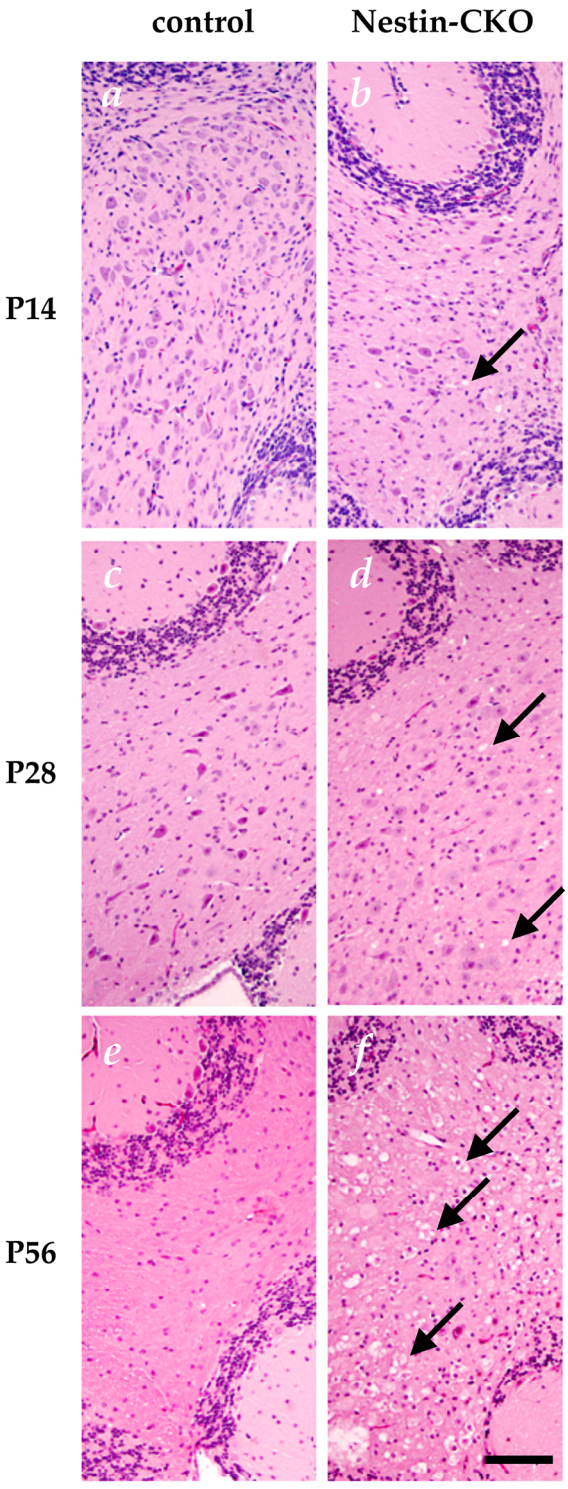

control
Nestin-CKO
a
b
c
d
e
f
EGL
ML
PCL
IGL
P14
ML
PCL
GL
P28
ML
PCL
GL
P56

Figure 2.8 | Deletion of FIP200 in cerebellum leads to progressive development of spongiform degeneration in Nestin-CKO mice. Cerebellar sections from control (*a*, *c*, *e*) and Nestin-CKO (*b*, *d*, *f*) mice at different ages were stained by H&E. Arrows mark spongiform degeneration in the white matter. Scale bar=200 μm.


control
Nestin-CKO
P14
P28
P56
a
b
c
d
e
f

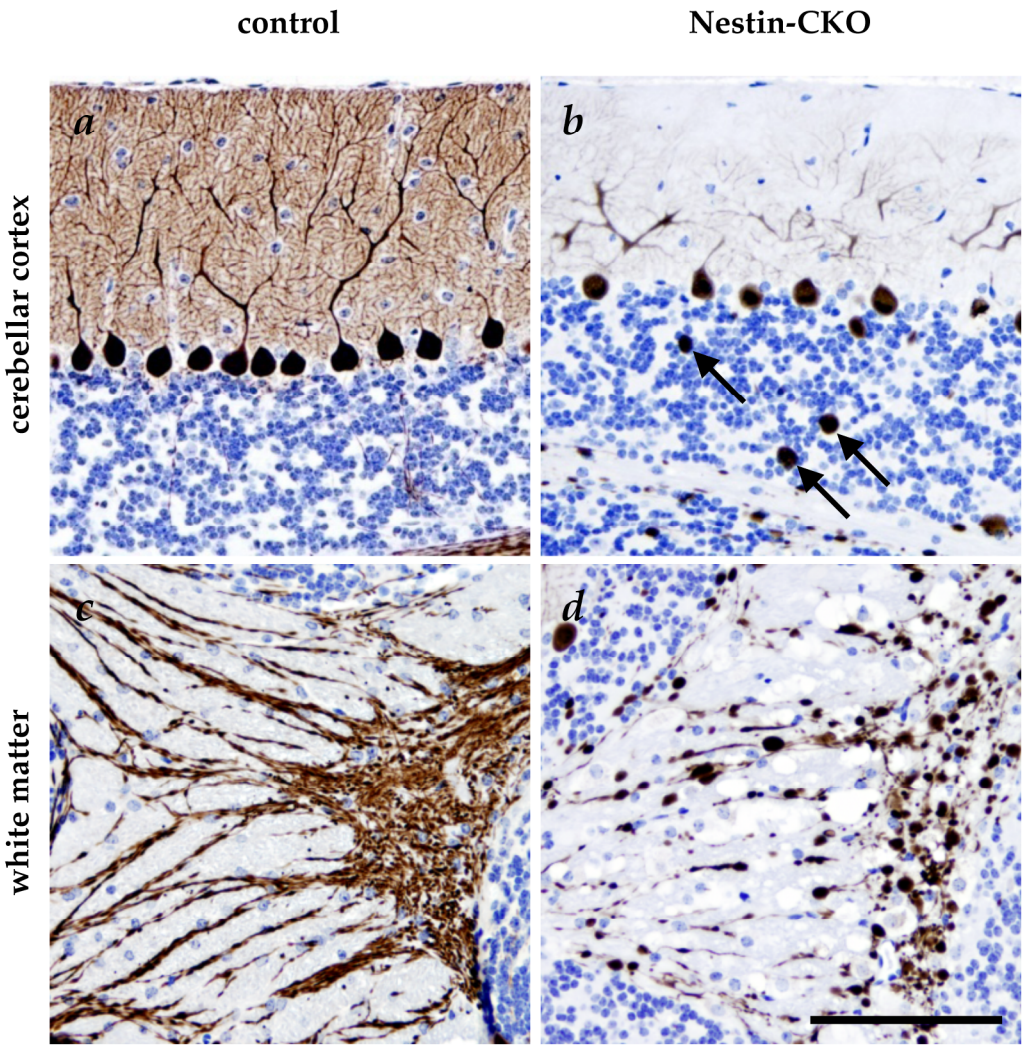


Figure 2.9 | Purkinje cell degeneration in cerebellum of Nestin-CKO mice. Cerebellum sections from 6-week-old control and Nestin-CKO mice were analyzed by immunohistochemistry with anti-calbindin. Note the loss of Purkinje cells in the Purkinje cell layer and degeneration of their dendrites (*b*) and axons (*d*) in cerebellum from Nestin-CKO mice compared to control mice. Arrows in panel *b* mark abnormal staining with anti-calbindin in the granular layer of the mutant mice. Scale bars=200 μm.

Examination of the white matter indicated extensive axonal degeneration associated with swelling regions of the labeled axons as well as vacuoles typical of spongiform degeneration as detected by H/E staining (see Figure 2.7) in the mutant mice (Figure 2.9 panels *c* and *d*).

I next examined myelination in the white matter of the mutant mice because nestin-Cre is active in glial cells and potential defects in oligodendrocytes and myelination could also contribute to the cerebellum ataxia defects in Nestin-CKO mice (Fraser et al., 2008). Cerebellar sections prepared from 4-week-old Nestin-CKO and control mice were analyzed by immunohistochemistry using antibody against myelin basic protein (anti-MBP). Extensive labeling with anti-MBP was found in the white matter of Nestin-CKO as well as control mice, suggesting that myelination was not significantly affected in the mutant mice (Figure 2.10 panels *a* and *b*). Staining of the sections with anti-olig2 (marker for oligodendrocytes) showed no significant distinction in the number of oligodendrocytes in Nestin-CKO and control mice (data not shown). Thus, axonal degeneration of Purkinje cells is unlikely a secondary consequence caused by deficiency of myelination in the mutant mice.

Analogous to H/E and calbindin staining of the white matter (Figure 2.8 and 2.9), significant spongiform degeneration was also evident by anti-MBP staining. Interestingly, the vacuoles appear to be embedded in the circle of MBP staining (Figure 2.10 *b*), suggesting these vacuoles may result from the degeneration of Purkinje cell axons encircled with myelin. The vacuoles are negative by Oil red O staining for lipids or periodic acid-Schiff (PAS) staining for polysaccharides (data not shown). The samples were then further examined by transmission electron microscopy of sections of white matter of

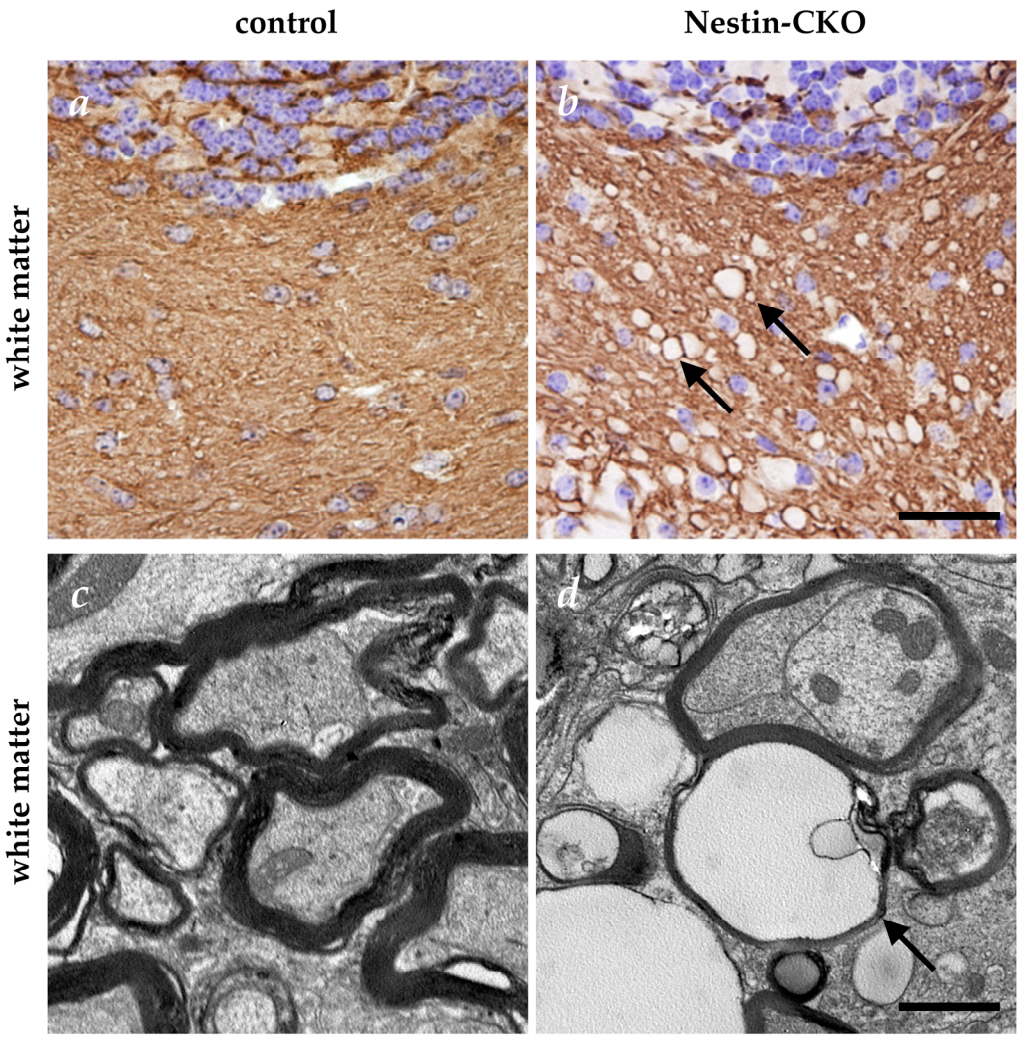


Figure 2.10 | Axon degeneration in cerebellum of Nestin-CKO mice. Cerebellum sections from 4-week-old control and Nestin-CKO mice were analyzed by immunohistochemistry with anti-MBP (panels *a* and *b*) or by transmission electron microscopy (panels *c* and *d*). Note that the vacuoles are embedded in myelin-sheath (arrows in *b* and *d*) of the mutant mice. Scale bars=50 μm (*a* and *b*) and 1 μm (*c* and *d*), respectively.

the mutant and control mice (Figure 2.10 panels *c* and *d*). Consistent with anti-MBP staining, apparently normal myelination was found in the mutant mice as well as control mice. However, the axon bundles in the mutant mice were more rounded compared to the irregular shape of that in the control mice, possibly as a consequence of swelling in some segments of the axons. Furthermore, some of the ensheathments did not contain axon fibers (arrow) in the mutant mice. These results are consistent with the MBP staining of the sections (see Figure 2.10 panels *a* and *b*) and provide further support for extensive swelling and degeneration of Purkinje cell axons, which may be responsible for the spongiform degeneration observed in the white matter of Nestin-CKO mice. Consistent with the extensive neural degeneration, immunostaining with antibody against GFAP showed significant reactive gliosis in the mutant cerebellum (Figure 2.11). Together, these studies suggested that reduced Purkinje cells and degeneration of their axons and dendrites are likely to be responsible for the loss of normal cerebellum functions in the motor coordination of Nestin-CKO mice.

2.3.3 Increased apoptosis and accumulation of ubiquitinated protein aggregates upon FIP200 deletion

To explore potential mechanisms by which deletion of FIP200 leads to neurodegeneration, I examined possible increases in apoptosis and ubiquitinated protein aggregates in the cerebellum of the mutant mice as many neurodegenerative diseases are associated with accumulation of ubiquitinated protein aggregates and loss of neurons resulting from apoptosis (Gao and Hu, 2008; Layfield et al., 2005; Rubinsztein, 2006). Previous studies showed that deletion of FIP200 resulted in increased apoptosis in the

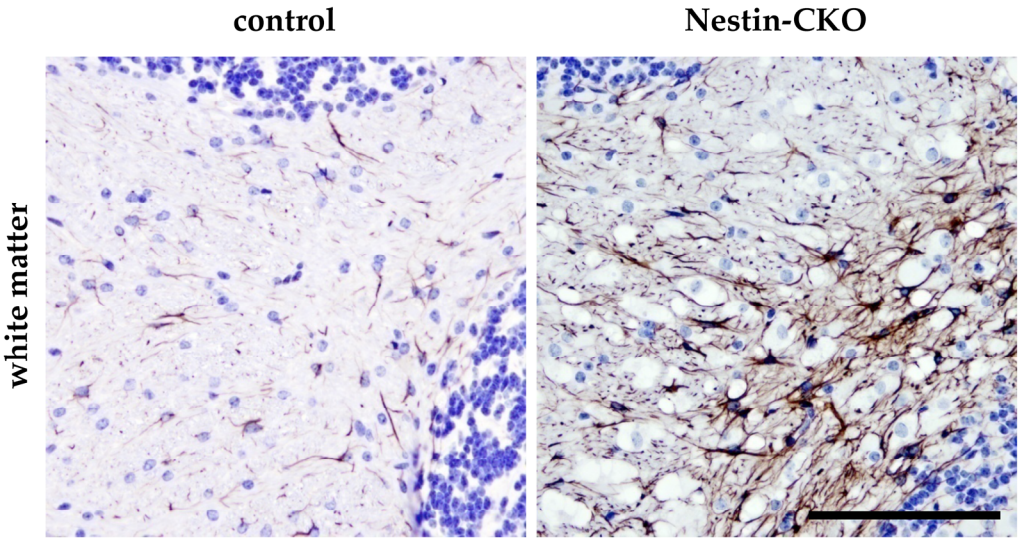


Figure 2.11 | Reactive gliosis in cerebellum of Nestin-CKO mice. Cerebellum sections from 6-week-old control and Nestin-CKO mice were analyzed by immunohistochemistry with anti-GFAP. Scale bar=200 μm.

developing heart and liver leading to major defects in these organs and embryonic lethality of KO mice (Gan et al., 2006). The observation of reactive gliosis also suggested the neuronal injury in the cerebellum in Nestin-CKO mice. I therefore examined whether an increased apoptosis may account for the reduced neurons in the Nestin-CKO mice by TUNEL assays of cerebella from the mutant and control mice at different postnatal days. Figure 2.12 shows that apoptosis was observed in the cerebella of control mice during the first 2 weeks of postnatal development as expected (Altman, 1972a; Altman, 1972b), but the mutant mice showed an increase in apoptosis compared to the controls at these times. As expected, the normal developmental apoptosis in the control mice reduced to a minimal level concomitant with the completion of postnatal development of the cerebellum at 3 weeks after birth. In contrast, significant levels of apoptosis were found in the mutant cerebella at 3 weeks and later after birth. These results suggest that depletion of FIP200 in the neurons could lead to increased apoptosis leading to neural degeneration in the mutant cerebellum.

I next examined the potential accumulation of ubiquitinated protein aggregates by staining of cerebellum sections from Nestin-CKO and control mice were with the anti-ubiquitin antibody. While no sign of ubiquitin aggregation was detected in the control cerebellum, large ubiquitin-positive aggregates were found in the white matter (Figure 2.13 panel *a* and *b*; arrows) and Purkinje cells (Figure 2.13 panel *e*; arrows) in the mutant cerebellum. Moreover when samples of Nestin-CKO cerebella were compared their immunoactivity of ubiquitin between different ages (Figure 2.13 panel *c* and *d*) I found that the small protein aggregates at P0 became the larger ones at P42,

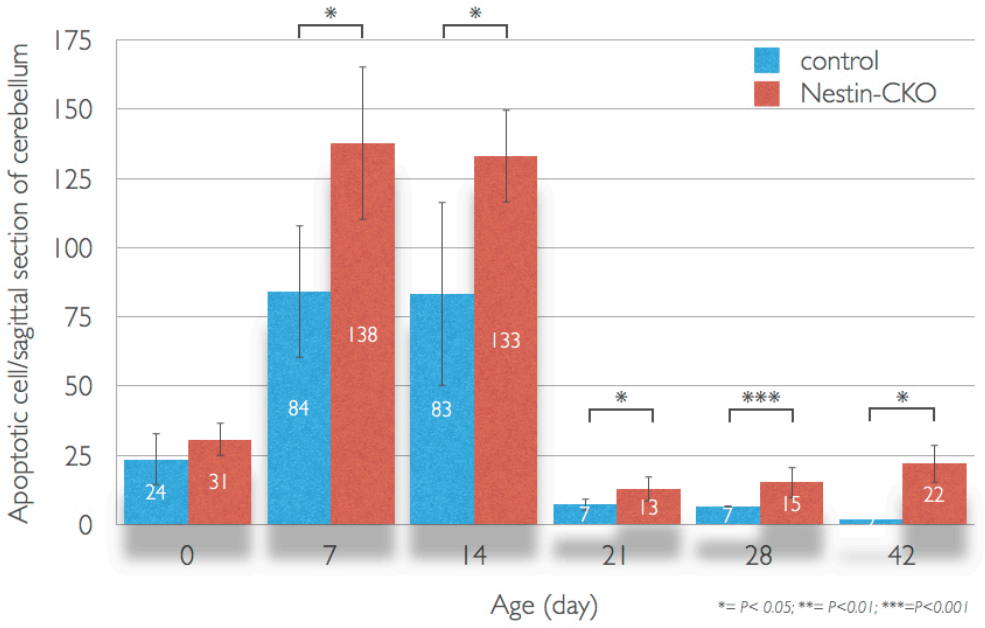
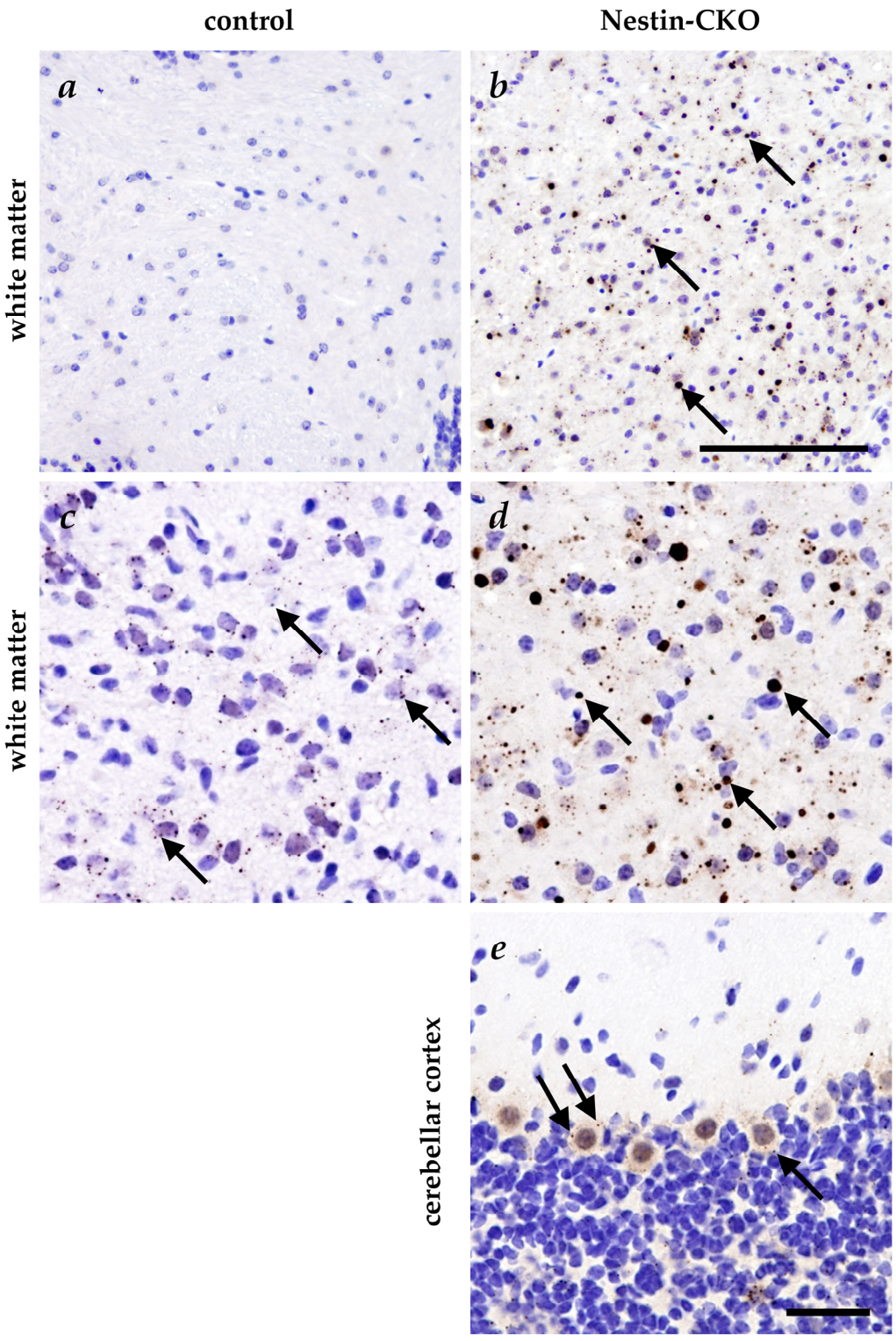


Figure 2.12 | Increased apoptosis in cerebellum of Nestin-CKO mice. Cerebellum sections from control or Nestin-CKO mice at various postnatal days were analyzed by TUNEL assays. The number of apoptotic cells were determined and the mean ± s.e. from three experiments is shown.

Figure 2.13 | Ubiquitinated protein aggregates in cerebellum of Nestin-CKO mice. Cerebellum sections from control or Nestin-CKO mice were analyzed by immunohistochemistry using anti-ubiquitin. Arrows marks the aggregates of ubiquitinated proteins. Note the ubiquitin-positive aggregates in the white matter at P42 (*b*) and in the Purkinje cells at P14 (*e*). The ubiquitinated protein aggregates progressively accumulated during aging (compare panels *c* at P0, and *d* at P42). Scale bars=200 μm (*a* and *b*) and 50 μm (*c*, *d*,and *e*), respectively.

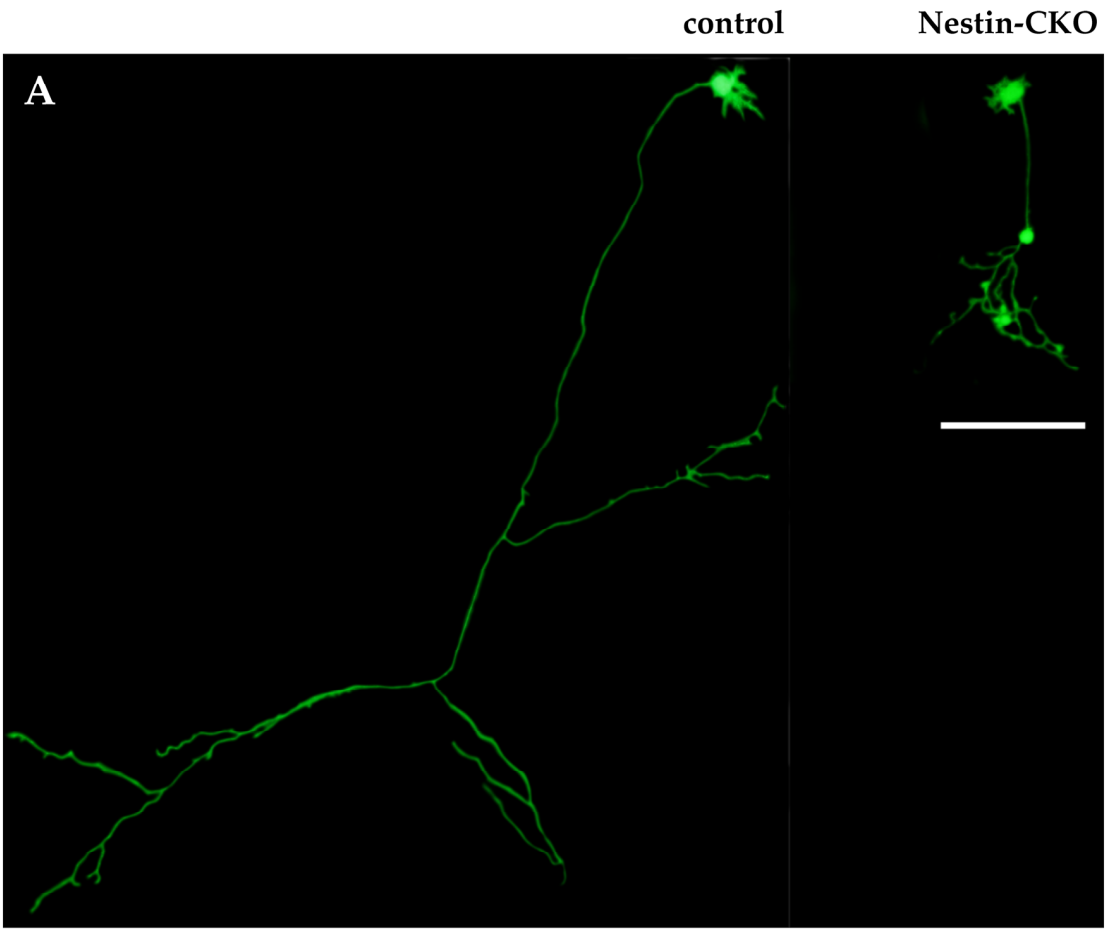

control
Nestin-CKO
a
b
white matter
c
d
white matter
e
cerebellar cortex

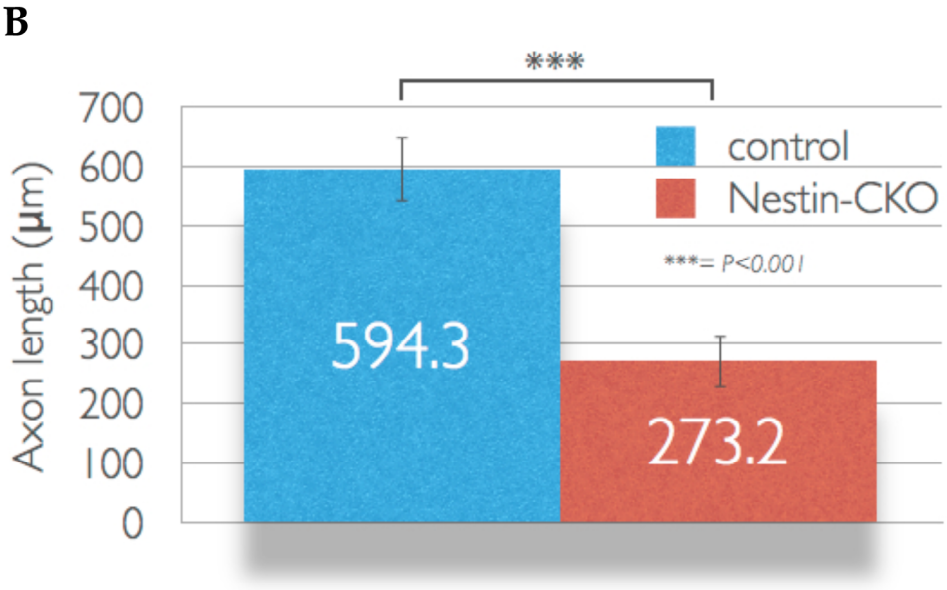
indicating that deletion of FIP200 resulted in abnormal progressive accumulation of ubiquitinated protein aggregates.

2.3.4 Deletion of FIP200 in the Purkinje cells led to axonopathy

From the previous immunohistochemistry stainings against calbindin, results have shown that the Purkinje cells in the Nestin-CKO cerebellum exhibit abnormal morphology, such as dendrite degeneration and axon swelling. To examine whether ablation of FIP200 causes axonopathy of the Purkine cells, the whole cerebellar cells from the Nestin-CKO or their control littermates was prepared and cultured in neuron-specific media for 7 days. *In vitro* neuronal cell cultures primarily contain two types of neurons, granule cells and Purkinje cells. After they were cultured 7 days *in vitro*, the Purkinje cells were fixed, analyzed by immunofluorescent using anti-calbindin, and observed the morphology by microscopy. As seen in Figure 2.14 (panel A), the Purkinje cells from the control mice grew, extended, and branched their thin axons to form synapses with other neuronal cells in the cell culture. In contrast, the Purkinje cells with FIP200-deletion exhibited axonopathy, including reduced axon outgrowth and numerous swollen segments of axon (Figure 2.14A and 2.15). The quantitative data showed the axonal outgrowth of the Purkinje cells from Nestin-CKO was significantly reduced approximately 60% as compared to their control cells (Figure 2.14, panel B). The *in vitro* results of axonophathy on the Purkinje cells were consistent with my *in vivo* findings by using immunohistochemistry against calbindin. These *in vitro* and *in vivo* results both suggested that ablation of FIP200 led to abnormal morphology of the Purkinje cells.

Figure 2.14 | Reduced axon outgrowth of the Nestin-CKO Purkinje cell. (A) *In vitro* Purkinje cells from control or Nestin-CKO mice at 7 days were analyzed by immunofluorecent using anti-calbindin. (B) The axon length of the Purkinje cells were determined and the mean ± s.e. from three experiments is shown (cell number=25). Scale Bar=100 μm.


control
Nestin-CKO
A


B
***
700
600
500
400
300
200
100
0
Axon length (μm)
control
Nestin-CKO
***= P<0.001
594.3
273.2

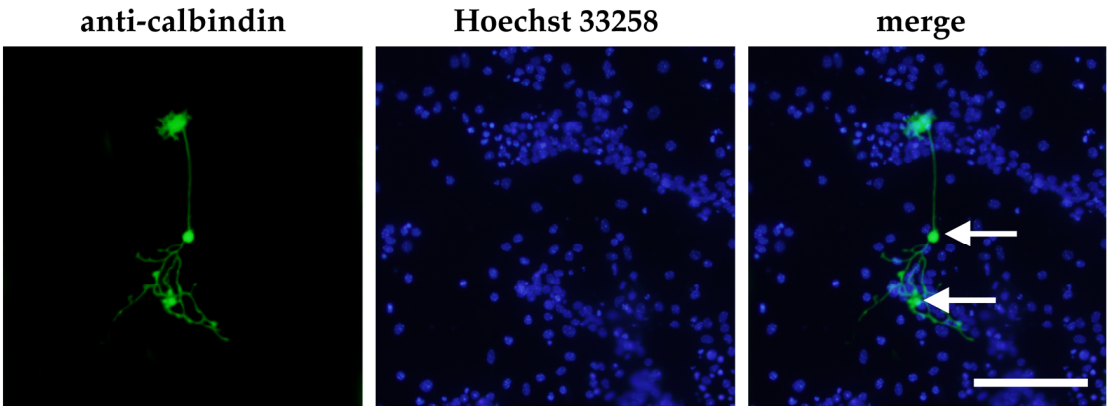


Figure 2.15 | Axonopathy of the Purkinje cell from the cerebellum of Nestin-CKO mice. *In vitro* Purkinje cells from control or Nestin-CKO mice at 7 days were analyzed by immunofluorecent using anti-calbindin and Hoechst 33258. Note arrows mark the swelling segment of axon. Scale Bars=100 μm.

2.4 Discussion

Work in the last decades has significantly advanced the understanding of the genetic basis of various neurodegenerative diseases by identifying mutations of genes in the diseases as well as the conceptualization that the accumulation of abnormal protein aggregation may be the underlying common mechanism of these diseases. Particularly, protein quality control mechanisms including the ubiquitin-proteasome system and autophagy have been suggested to play crucial roles in the homeostasis of neurons and neurodegeneration (Giobbe et al., 1991; Winslow and Rubinsztein, 2008). However, the molecular components and signaling pathways that regulate these cellular processes in various neurodegenerative diseases are still not well understood at present.

By using a neural-specific conditional KO approach, I identified FIP200 as an important regulatory protein in CNS for neuronal homeostasis. Deletion of FIP200 resulted in abnormal accumulation of ubiquitinated protein aggregates, axonal swelling, increased apoptosis and loss of Purkinje cells, spongiform degeneration in the cerebellum, which are associated with cerebellar degeneration and ataxia in mice. My studies showed that the mutant mice with conditional FIP200 deletion share several phenotypes as the recently reported Atg5 or Atg7 conditional KO mice by nestin-Cre (Hara et al., 2006; Komatsu et al., 2006), such as the increased ubiquitin aggregates, progressive loss of the Purkinje cells, increased apoptosis in the cerebellum, cerebellar ataxia, and early death of the mice. However, whether FIP200 is involved in the regulation of autophagy remains obscure presently.

Interestingly, a recent study identified an interaction between FIP200 and ULK1, mammalian homolog of yeast Atg1, and showed that FIP200 is

required for both mTOR-dependent and –independent autophagy in mammalian cells *in vitro* (Hara et al., 2008). Thus, the data presented here strongly suggest a role of FIP200 in the regulation of autophagy *in vivo* and deficiency in autophagy upon FIP200 inactivation is likely a major contributor to the neurodegeneration in the mutant mice. Although ULK1 has been shown to be mammalian ortholog of yeast Atg1 in the Atg1/Atg13/Atg17 complex critical for autophagosome initiation, the mammalian homologs for the other two components have not been characterized. Aside from the previous report on FIP200 interaction with ULK1, studies have shown that a recent identified mammalian ortholog of Atg13 interacts with both ULK1 and FIP200 in a high molecular weight complex (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009). With these *in vitro* data, they provide further support for the idea that FIP200 is likely a mammalian functional counterpart of Atg17 despite limited homology in their protein sequences (Hara and Mizushima, 2009). While they share many phenotypes with Atg5 or Atg7 conditional KO, FIP200 conditional KO mice developed many defects such as loss of Purkinje cells as well as movement disorders at an earlier age. As ULK1-Atg13-FIP200 complex is required for autophagosome initiation whereas Atg5 and Atg7 are involved in the later steps of autophagy (Hara et al., 2006; Komatsu et al., 2006), it is possible that these differences reflect the temporal requirements for autophagy to remove abnormal protein aggregates that were responsible for the various neural degeneration phenotypes. On the other hand, FIP200 has been shown to regulate several other signaling pathways and cellular processes besides its interaction with ULK1 and regulation of autophagy (Hara et al., 2008). It is therefore possible that perturbations of other signaling

pathways may contribute to the earlier and maybe severer phenotypes in the FIP200 conditional KO mice.

My studies also revealed several distinctive features of neural-specific FIP200 conditional KO mice including the progressive development of spongiform degeneration that were not observed in the Atg5 or Atg7 conditional KO mice (Hara et al., 2006; Komatsu et al., 2006). Signs of spongiform degeneration in the white matter were found as early as 2 weeks of age in Nestin-CKO mice and precede the loss of Purkinje cells in the mutant mice. Furthermore, I also observed the swelling of segments of Purkinje cell axons both *in vivo* (see Figure 2.9) and *in vitro* (see Figure 2.15). The blockages in axonal transport resulting from defects in microtubule based motors has been proposed to be responsible for the axonal swelling phenotype (Bendiske and Bahr, 2003; Stokin et al., 2005). Thus, it is possible that ablation of FIP200 could induce the neuronal defects because of abnormality in the axonal transport machinery in Purkinje cells, independent of (or in combination with) the potential autophagy defects discussed above. Alternatively, such blockages could be caused by the progressive accumulation of ubiquitinated protein aggregates (instead of the transport machinery itself) upon FIP200 deletion but not (or not as severe) in Atg5- or Atg7-deficient neurons. In this regard, it is interesting to note that the aggregation of ubiquitinated proteins was first observed in the white matter correlated with the early signs of spongiform degeneration and anterior to any apparent loss of Purkinje cells. Therefore, the progressive degeneration of Purkinje cell axons may be responsible for the dysfunction of these neurons and their death.

Spongiform degeneration is caused by extensive vacuolization of neuronal cells, which are typically associated with brain damage induced by

Prions (Hooper, 2003). The underlying mechanism of such vacuolization is not well understood but mitochondrial dysfunction and increased reactive oxygen species (ROS) production and/or sensitive to ROS have been suggested to be responsible for spongiform degeneration in several disease models (Akassoglou et al., 2004; Li et al., 1995; Lin et al., 2004; Matalon et al., 2000; Puccio et al., 2001). Interestingly, several other mice KO models of neurodegenerative diseases such as those with deletions in mitochondrial protein, Sod1 (Li et al., 1995) or PGC1-α (Lin et al., 2004), also showed spongiform degeneration as prominent features of the neurodegeneration phenotype. It will be interesting to determine potential abnormalities in mitochondria as well as ROS production and/or sensitivity upon deletion of FIP200 in neurons or other cell types, which may or may not be caused by abnormal accumulation of ubiquitinated protein aggregates. The above considerations suggest that deletion of FIP200 in neurons could induce axon swelling and/or spongiform degeneration because of deficiencies in other signaling pathways rather than (or in combination with) defects in autophagy that are present in Atg5 or Atg7 conditional KO mice.

Consistent with this possibility, previous studies showed that total KO of FIP200 caused embryonic lethality resulting from defective heart and liver development as a result of defective TNFα-JNK and TSC-mTOR signaling pathways (Gan et al., 2006), whereas *Atg5*$^{-/-}$ and *Atg7*$^{-/-}$ mice were born at normal Mendalian ratio and dies only shortly after birth because of deficiency in autophagy (Hara et al., 2006; Komatsu et al., 2006). It is interesting to note that both FIP200 KO MEFs and liver cells have shown increased sensitivity to TNFα-induced cell death compared to the control cells (Gan et al., 2006). Although a reduction in JNK-mediated survival signaling was shown to be

responsible for the increased apoptosis in these cells upon TNFα treatment, it is known that TNFα stimulation also induces production of ROS which could play a role in cell survival and death. Thus, it is conceivable that an abnormality in ROS production and/or sensitivity in neuronal cells upon FIP200 could contribute to the axonal swelling leading to spongiform degeneration in the mutant mice. mTOR signaling pathways have also been implicated in the regulation neurodegeneration, neuronal cell survival, and axonal development (Jaworski and Sheng, 2006). Consistent with previous studies in cells and embryos (Gan et al., 2006), reduced mTOR activity were found in Purkinje cells of Nestin-CKO mice (data not shown), which could contribute to defects not seen in Atg5 or Atg7 conditional KO mice resulting from autophagy deficiency. Future studies will be required to resolve the potential contributions of these and possibly other signaling pathways in mediating neurodegenerative defects in FIP200 conditional KO mice.

REFERENCES

Akassoglou, K., B. Malester, J. Xu, L. Tessarollo, J. Rosenbluth, and M.V. Chao. 2004. Brain-specific deletion of neuropathy target esterase/swisscheese results in neurodegeneration. *Proc Natl Acad Sci U S A*. 101:5075-80.

Altman, J. 1972a. Postnatal development of the cerebellar cortex in the rat. 3. Maturation of the components of the granular layer. *J Comp Neurol*. 145:465-513.

Altman, J. 1972b. Postnatal development of the cerebellar cortex in the rat. I. The external germinal layer and the transitional molecular layer. *J Comp Neurol*. 145:353-97.

Bamba, N., T. Chano, T. Taga, S. Ohta, Y. Takeuchi, and H. Okabe. 2004. Expression and regulation of RB1CC1 in developing murine and human tissues. *Int J Mol Med*. 14:583-7.

Bates, B., M. Rios, A. Trumpp, C. Chen, G. Fan, J.M. Bishop, and R. Jaenisch. 1999. Neurotrophin-3 is required for proper cerebellar development. *Nat Neurosci*. 2:115-7.

Bendiske, J., and B.A. Bahr. 2003. Lysosomal activation is a compensatory response against protein accumulation and associated synaptopathogenesis--an approach for slowing Alzheimer disease? *J Neuropathol Exp Neurol*. 62:451-63.

Chano, T., S. Ikegawa, F. Saito-Ohara, J. Inazawa, A. Mabuchi, Y. Saeki, and H. Okabe. 2002. Isolation, characterization and mapping of the mouse and human RB1CC1 genes. *Gene*. 291:29-34.

Chano, T., H. Okabe, and C.M. Hulette. 2007. RB1CC1 insufficiency causes neuronal atrophy through mTOR signaling alteration and involved in the pathology of Alzheimer's diseases. *Brain Res*. 1168:97-105.

Dahlstrand, J., M. Lardelli, and U. Lendahl. 1995. Nestin mRNA expression correlates with the central nervous system progenitor cell state in many, but not all, regions of developing central nervous system. *Brain Res Dev Brain Res*. 84:109-29.

Frappart, P.O., Y. Lee, J. Lamont, and P.J. McKinnon. 2007. BRCA2 is required for neurogenesis and suppression of medulloblastoma. *EMBO J*. 26:2732-42.

Fraser, M.M., I.T. Bayazitov, S.S. Zakharenko, and S.J. Baker. 2008. Phosphatase and tensin homolog, deleted on chromosome 10 deficiency in brain causes defects in synaptic structure, transmission and plasticity, and myelination abnormalities. *Neuroscience*. 151:476-88.

Gan, B., and J.L. Guan. 2008. FIP200, a key signaling node to coordinately regulate various cellular processes. *Cell Signal*. 20:787-94.

Gan, B., X. Peng, T. Nagy, A. Alcaraz, H. Gu, and J.L. Guan. 2006. Role of FIP200 in cardiac and liver development and its regulation of TNFalpha and TSC-mTOR signaling pathways. *J Cell Biol*. 175:121-33.

Ganley, I.G., D.H. Lam, J. Wang, X. Ding, S. Chen, and X. Jiang. 2009. ULK1-ATG13-FIP200 complex mediates mTOR signaling and is essential for autophagy. *J Biol Chem*.

Gao, X., and H. Hu. 2008. Quality control of the proteins associated with neurodegenerative diseases. *Acta Biochim Biophys Sin (Shanghai)*. 40:612-8.

Giobbe, C., R. Piacentino, R. Grio, G.L. Marchino, F. Zaccheo, O. Mazza, D. Malara, G. Baccarini, and L. Ferrara. 1991. [Clinical aspects of endometriosis]. *Minerva Ginecol*. 43:1-5.

Hara, T., and N. Mizushima. 2009. Role of ULK-FIP200 complex in mammalian autophagy: FIP200, a counterpart of yeast Atg17? *Autophagy*. 5:85-7.

Hara, T., K. Nakamura, M. Matsui, A. Yamamoto, Y. Nakahara, R. Suzuki-Migishima, M. Yokoyama, K. Mishima, I. Saito, H. Okano, and N. Mizushima. 2006. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature*. 441:885-9.

Hara, T., A. Takamura, C. Kishi, S. Iemura, T. Natsume, J.L. Guan, and N. Mizushima. 2008. FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells. *J Cell Biol*. 181:497-510.

Hooper, N.M. 2003. Could inhibition of the proteasome cause mad cow disease? *Trends Biotechnol*. 21:144-5.

Hosokawa, N., T. Hara, T. Kaizuka, C. Kishi, A. Takamura, Y. Miura, S.I. Iemura, T. Natsume, K. Takehana, N. Yamada, J.L. Guan, N. Oshiro, and N. Mizushima. 2009. Nutrient-dependent mTORC1 Association with the ULK1-Atg13-FIP200 Complex Required for Autophagy. *Mol Biol Cell*.

Jaworski, J., and M. Sheng. 2006. The growing role of mTOR in neuronal development and plasticity. *Mol Neurobiol*. 34:205-19.

Jung, C.H., C.B. Jun, S.H. Ro, Y.M. Kim, N.M. Otto, J. Cao, M. Kundu, and D.H. Kim. 2009. ULK-Atg13-FIP200 Complexes Mediate mTOR Signaling to the Autophagy Machinery. *Mol Biol Cell*.

Kandel, E.R., J.H. Schwartz, and T.M. Jessell. 2000. Principles of neural science. McGraw-Hill, Health Professions Division, New York. xli, 1414 p. pp.

Komatsu, M., S. Waguri, T. Chiba, S. Murata, J. Iwata, I. Tanida, T. Ueno, M. Koike, Y. Uchiyama, E. Kominami, and K. Tanaka. 2006. Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature*. 441:880-4.

Layfield, R., J. Lowe, and L. Bedford. 2005. The ubiquitin-proteasome system and neurodegenerative disorders. *Essays Biochem*. 41:157-71.

Li, Y., T.T. Huang, E.J. Carlson, S. Melov, P.C. Ursell, J.L. Olson, L.J. Noble, M.P. Yoshimura, C. Berger, P.H. Chan, D.C. Wallace, and C.J. Epstein. 1995. Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet*. 11:376-81.

Lin, J., P.H. Wu, P.T. Tarr, K.S. Lindenberg, J. St-Pierre, C.Y. Zhang, V.K. Mootha, S. Jager, C.R. Vianna, R.M. Reznick, L. Cui, M. Manieri, M.X. Donovan, Z. Wu, M.P. Cooper, M.C. Fan, L.M. Rohas, A.M. Zavacki, S. Cinti, G.I. Shulman, B.B. Lowell, D. Krainc, and B.M. Spiegelman. 2004. Defects in adaptive energy metabolism with CNS-linked hyperactivity in PGC-1alpha null mice. *Cell*. 119:121-35.

Matalon, R., P.L. Rady, K.A. Platt, H.B. Skinner, M.J. Quast, G.A. Campbell, K. Matalon, J.D. Ceci, S.K. Tyring, M. Nehls, S. Surendran, J. Wei, E.L. Ezell, and S. Szucs. 2000. Knock-out mouse for Canavan disease: a model for gene transfer to the central nervous system. *J Gene Med*. 2:165-75.

Puccio, H., D. Simon, M. Cossee, P. Criqui-Filipe, F. Tiziano, J. Melki, C. Hindelang, R. Matyas, P. Rustin, and M. Koenig. 2001. Mouse models for Friedreich ataxia exhibit cardiomyopathy, sensory nerve defect and

Fe-S enzyme deficiency followed by intramitochondrial iron deposits. *Nat Genet*. 27:181-6.

Ross, C.A., and M.A. Poirier. 2004. Protein aggregation and neurodegenerative disease. *Nat Med*. 10 Suppl:S10-7.

Rubinsztein, D.C. 2006. The roles of intracellular protein-degradation pathways in neurodegeneration. *Nature*. 443:780-6.

Stokin, G.B., C. Lillo, T.L. Falzone, R.G. Brusch, E. Rockenstein, S.L. Mount, R. Raman, P. Davies, E. Masliah, D.S. Williams, and L.S. Goldstein. 2005. Axonopathy and transport deficits early in the pathogenesis of Alzheimer's disease. *Science*. 307:1282-8.

Tronche, F., C. Kellendonk, O. Kretz, P. Gass, K. Anlag, P.C. Orban, R. Bock, R. Klein, and G. Schutz. 1999. Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat Genet*. 23:99-103.

Ueda, H., S. Abbi, C. Zheng, and J.L. Guan. 2000. Suppression of Pyk2 kinase and cellular activities by FIP200. *J Cell Biol*. 149:423-30.

Wei, H., B. Gan, X. Wu, and J.L. Guan. 2009. Inactivation of FIP200 Leads to Inflammatory Skin Disorder, but Not Tumorigenesis, in Conditional Knock-out Mouse Models. *J Biol Chem*. 284:6004-13.

Winslow, A.R., and D.C. Rubinsztein. 2008. Autophagy in neurodegeneration and development. *Biochim Biophys Acta*. 1782:723-9.

Chapter 3
NEURAL SPECIFIC DELETION of FIP200 LEADS to CEREBELLAR DEGENERATION THROUGH DEFECTIVE AUTOPHAGY AND ALTERED TNFR-1 SIGNALING PATHWAY

3.1 Introduction

Damages to the cerebellum caused by either injury or neurodegeneration are associated with ataxia both in humans as well as mouse models (Goldowitz and Hamre, 1998; Sillitoe and Joyner, 2007; Wang and Zoghbi, 2001). Previous studies have identified various gene mutations underlying the different neurodegenerative disorders and revealed the accumulation of abnormal protein aggregation as a potentially common cellular mechanism of these diseases. However, our understanding of the molecular and cellular mechanisms by which key signaling molecules and pathways regulate these cellular processes in neurodegeneration is still incomplete. FIP200 is an evolutionarily conserved 200 kDa protein characterized by a large coiled-coil region (Chano et al., 2002b; Ueda et al., 2000). Initially identified as a protein that interacts with and inhibits the kinases Pyk2 and FAK (Ueda et al., 2000), FIP200 also associates with other cellular proteins and regulates several signaling pathways (Gan and Guan, 2008).

Autophagy is an evolutionarily conserved cellular process from yeast to man (Cuervo, 2004; Levine and Klionsky, 2004; Mizushima, 2007; Mizushima and Klionsky, 2007; Mizushima et al., 2008; Rubinsztein, 2006), that plays essential roles in the removal of proteins with aberrant structures to maintain cellular homeostasis, which is particularly important in post-mitotic cells such as neurons. Genetic studies using yeast as a model system have identified

thirty-one autophagy-related ATG genes and defined the complex formations of the Atg proteins and their functions (Klionsky, 2005; Suzuki and Ohsumi, 2007). Mammalian orthologs for some of the yeast Atg proteins have been identified and characterized to play crucial roles in autophagy in development and diseases processes such as cancer and neurodegeneration (Hippert et al., 2006; Winslow and Rubinsztein, 2008). Aside from the recent findings suggesting a role of FIP200 in autophagosome initiation as a key component of the ULK1-Atg13-FIP200 complex in fibroblasts (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009), RNAi-mediated knockdown of FIP200 has been shown to cause neurite atrophy and apoptosis in a neuroblastoma cell line Neuro-2a (Chano et al., 2007; Chano et al., 2006). However, it remains unknown about the potential role and mechanisms of FIP200 regulation of autophagy and homeostasis of neurons *in vivo*. Although the primary function of autophagy is the supply of amino acids as a response to starvation conditions in many organisms, basal autophagy, independent of nutrient stress, also has been suggested that it plays an important role in maintaining cellular homeostasis, particularly in quiescent cells such as neurons. FIP200 was recently proposed as a mammalian counterpart of yeast Atg17 (autophagy-related 17) required for autophagosome initiation despite its different structure and sequence from Atg17 (Hara and Mizushima, 2009).

To study whether mammalian FIP200 regulates autophagy *in vivo* and whether its regulation of autophagosome initiation or other signaling pathways is involved in neuronal homeostasis and neurodegeneration, I generated transgenic mice with conditional deletion of FIP200 using nestin-Cre, hGFAP-Cre and L7-Cre with complementary Cre expression in the cerebellum provided support for a Purkinje cell-autonomous function of

FIP200 in the regulation of cerebellar degeneration. Consistent with a role of FIP200 in autophagy, I observed progressive accumulation of abnormal ubiquitinated protein aggregates without any impairment of the ubiquitin-proteasome activity, increased apoptosis, and mitochondrial damage in Purkinje cells of mutant mice. Lastly, I found that deletion of TNFR-1 rescued both the loss of Purkinje cells and spongiform degeneration in FIP200 conditional KO mice. Together, these results provide compelling genetic evidence that FIP200 regulation of autophagy and TNFR-1 signaling plays critical roles in the pathogenesis of neurodegenerative disorders in mammals.

3.2 Material and Methods

Animals and genotyping

FIP200$^{flox/flox}$ mice were described previously (Gan et al., 2006). Nestin-Cre (Tronche et al., 1999), hGFAP-Cre (Zhuo et al., 2001), L7-Cre (Smeyne et al., 1995) and TNFR-1 (Pfeffer et al., 1993) transgenic mice were obtained from The Jackson Laboratory (Bar Harbor, ME). *p53*$^{flox/flox}$ transgenic mice (Jonkers et al., 2001) were kind gifts from Dr. Alexander Nikitin (Cornell University). Mice were housed and handled according to local, state, and federal regulations, and all experimental procedures were carried out according to the guidelines of Institutional Animal Care and Use Committee at Cornell University and the University of Michigan. Mice genotyping for FIP200 and Cre alleles were performed by PCR analysis of tail DNA, essentially as described previously (Gan et al., 2006; Wei et al., 2009).

Histology and Immunohistochemistry

Mice were euthanized using CO_2, and a complete tissue set was harvested

during necropsy. Fixation was carried out for 16 h at 4°C using freshly made, pre-chilled (4°C) PBS-buffered formalin. The brain tissues were all sagittal sectioned and then embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin for routine histological examination or left unstained for immunohistochemistry. Hematoxylin- and eosin-stained sections were examined under an Olympus BX41 light microscope (Olympus America, Inc., Center Valley, PA), and images were captured with an Olympus digital camera (model DP70) using a DP Controller software (Version 1.2.1.10 [EC] 8). For immunohistochemistry, unstained tissue sections were first deparaffinized in 3 washes of xylene (3 min each) and were rehydrated in graded ethanol solutions (100, 95, 70, 50, and 30%). After heat-activated antigen retrieval (model Retriever 2000, PickCellLaboratories B.V., Amsterdam, Holland) according to manufacturer's specifications, sections were treated with blocking solutions; first with Avidin-Biotin Block (Dako Corp., Carpinteria, CA) then with Protein Block Serum Free (Dako Corp.). Sections were then incubated with the primary antibody (calbindin 1:2000, Sigma-Aldrich; ubiquitin 1:500, Cell signaling; Dako corp.; cytochrome c 1:100 BD) at 37 °C for 1 h in a humid chamber, washed in PBS 3 times (5 min each), then incubated with the biotinylated secondary antibody anti-mouse for calbindin, ubiquitin, and cytochrome c (1:200 dilution, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in a humidified chamber for 1 h at 37 °C and washed in PBS similarly as before. Finally, sections were incubated with horseradish peroxidase-streptavidin (ABC Elite kit, Vector Laboratories) for 15 min at room temperature in a humid chamber and washed with PBS similarly as before. As the last staining step, 3,3'-diaminobenzidine (SIGMA FAST® DAB with Metal Enhancer, Sigma-Aldrich) was added to the sections and incubated at room

temperature until a macroscopically appreciable light brown color developed inthe sections (generally 30 s to 5 min). After incubation with DAB, sections were lightly counterstained will Gill's hematoxylin. Histological examination and digital photography were carried out as described previously.

Proteasomal catalytic activity assay

Proteasomal catalytic activity was analyzed for the lysates using the synthetic peptide substrates linked to the fluorometric receptor and aninomethylcoumarin (AMC) (Proteasome Substrate Pack from Biomol, Plymouth Meeting, PA). Mice cerebella were dissected, placed on ice and homogenized in proteolysis activity buffer (DTT 0.5 mM, ATP 5 mM, $MgCl_2$ 5 mM). The 250 µl aliquots containing equal amounts of protein were incubated for 30 min at 37 °C with 2.5 µl of Ac-Gly-Pro-Leu-Asp-AMC (5 mM), Z-Leu-Leu-Glu-AMC (5 mM), Suc-Leu-Leu-Val-Tyr-AMC (5 mM), Ac-Arg-Leu-Arg-AMC (5 mM) or Boc-Leu-Arg-Arg-AMC (5 mM) for caspase-like, chymotrypsin or trypsin-like activity, respectively. The reaction was stopped by adding 252.5 µl ice-cold ethanol (96%). The Proteasomal catalytic activity was determined by measuring the increase of fluorescence from AMC hydrolysis (380 nm excitation and 460 nm emission).

Transmission electron microscopy

Samples were fixed in 2.5% glutaraldehyde in 0.1 M Sorensen's buffer, pH 7.4, overnight at 4°C. After several rinses with buffer, they were postfixed in 1% osmium tetroxide in the same buffer. They were then rinsed in double distilled water to remove phosphate salt and then stained with aqueous 3% uranyl acetate for one hour. The samples were dehydrated in ascending

concentrations of ethanol, rinsed two times in propolynoxide, and embedded in epoxy resin. They were ultra-thin sectioned at 70 nm in thickness and stained with uranyl acetate and lead citrate. The sections were examined using a Philips CM100 electron microscope at 60 kV. Images were recorded digitally using an Hamamatsu ORCA-HR digital camera system operated using AMT software (Advanced Microscopy Techniques Corp., Danvers, MA).

Rotarod test

The Rotarod experiments were performed as described previously (McKinney and Murphy, 2006). Mice were placed on the rotating drum of an accelerating rotarod (Ugo Basile/Stoelting accelerating rotarod, Chicago, IL, USA). During a 5-min accelerating period of the rotarod speed from 4 to 40 rpm, the time for each mouse staying on top of the drum was recorded.

3.3 Results

3.3.1 Analysis of conditional KO of FIP200 in cerebellar neurons other than Purkinje cells using hGFAP-Cre

As delineated in Chapter 2, nestin-Cre is active in the neural precursors which give rise to both cerebellar neurons and glial cells (Bates et al., 1999; Dahlstrand et al., 1995; Frappart et al., 2007), raising the possibility that deficiency in other neurons and/or glial cells of the cerebellum could also contribute to the ataxia phenotype either directly or indirectly by causing secondary degenerative defects of Purkinje cells (Baptista et al., 1994; Morrison and Mason, 1998). Although my analysis of Nestin-CKO mice showed apparently normal myelination (see Figure 2.10) as well as oligodendrocytes (data not shown), loss of granule cells and interneurons are

noted in these mice (see Figure 2.7). Therefore, to study a possible Purkinje cell-autonomous function of FIP200 in cerebellar degeneration and ataxia, I created FIP200 conditional KO mice using hGFAP-Cre mice (designated as hGFAP-CKO mice) which express Cre in cerebellar neurons as well as glial cells, but not in Purkinje cells (Zhuo et al., 2001). Consistent with the more restricted expression pattern of hGFAP-Cre compared to that of nestin-Cre, hGFAP-CKO mice were obtained at the expected Mendelian ratio. In contrast to the cerebellar ataxia phenotype starting at about 2 weeks of age and becoming very evident by 4 weeks as well as pre-mature lethality of Nestin-CKO mice by about 8 weeks of age (see Figure 2.1), hGFAP-CKO mice appeared healthy with apparently normal movements until at least 8 weeks of age. Histological examination of cerebellar sections prepared from hGFAP-CKO mice at 4 weeks of age showed a reduced number of interneurons in the molecular layer and a slight decrease of granule cells in the mutant mice compared to the control mice (Figure 3.1, compare panels *a* and *b*). In contrast to Nestin-CKO mice (panel *c*), however, I did not observe any defects in Purkinje cells and their dendrites extending into the molecular layer (arrows) or the reduction in the thickness of the molecular layer in hGFAP-CKO mice at 4 weeks of age (panel *b*). Staining of the sections with anti-calbindin showed apparently normal axons of Purkinje cells in hGFAP-CKO mice (Figure 3.2, compare panels *a* and *b*) in contrast to that in Nestin-CKO mice (panel *c*). These results are consistent with the lack of hGFAP-Cre expression in Purkinje cells and suggest that cerebellar degeneration and the ataxia phenotype observed in Nestin-CKO mice were caused by Purkinje cell-autonomous defects upon FIP200 deletion in these cells.

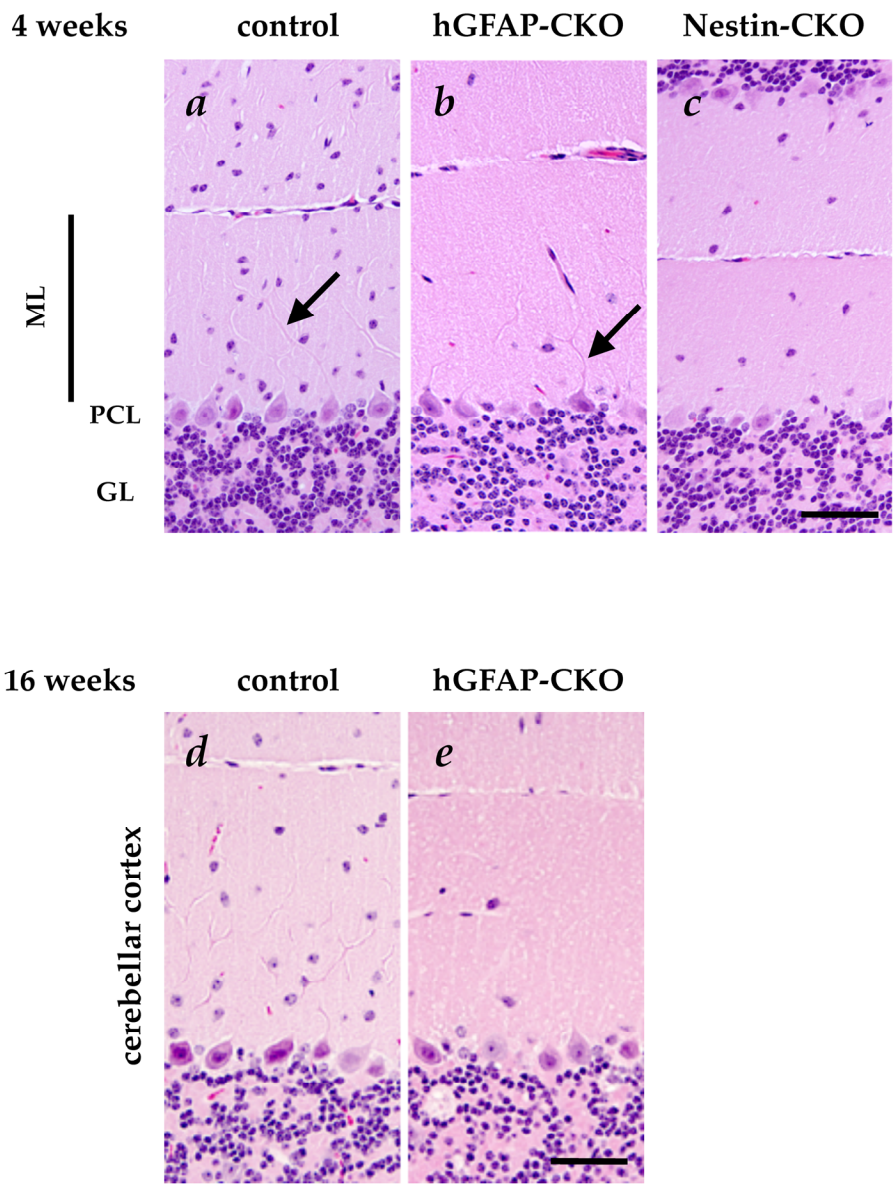


Figure 3.1 | Cerebellum cortex in hGFAP-CKO mice. Cerebellar sections from control (*a*, *d*), hGFAP-CKO (*b*, *e*) and Nestin-CKO mice (*c*) at different ages were stained by H&E. Molecular layer (ML), Purkinje cell layer (PCL) and internal granular layer (GL) are marked on the left. Arrows (*a* and *b*) mark normal dendrites from Purkinje cells. Scale bars=100 μm.

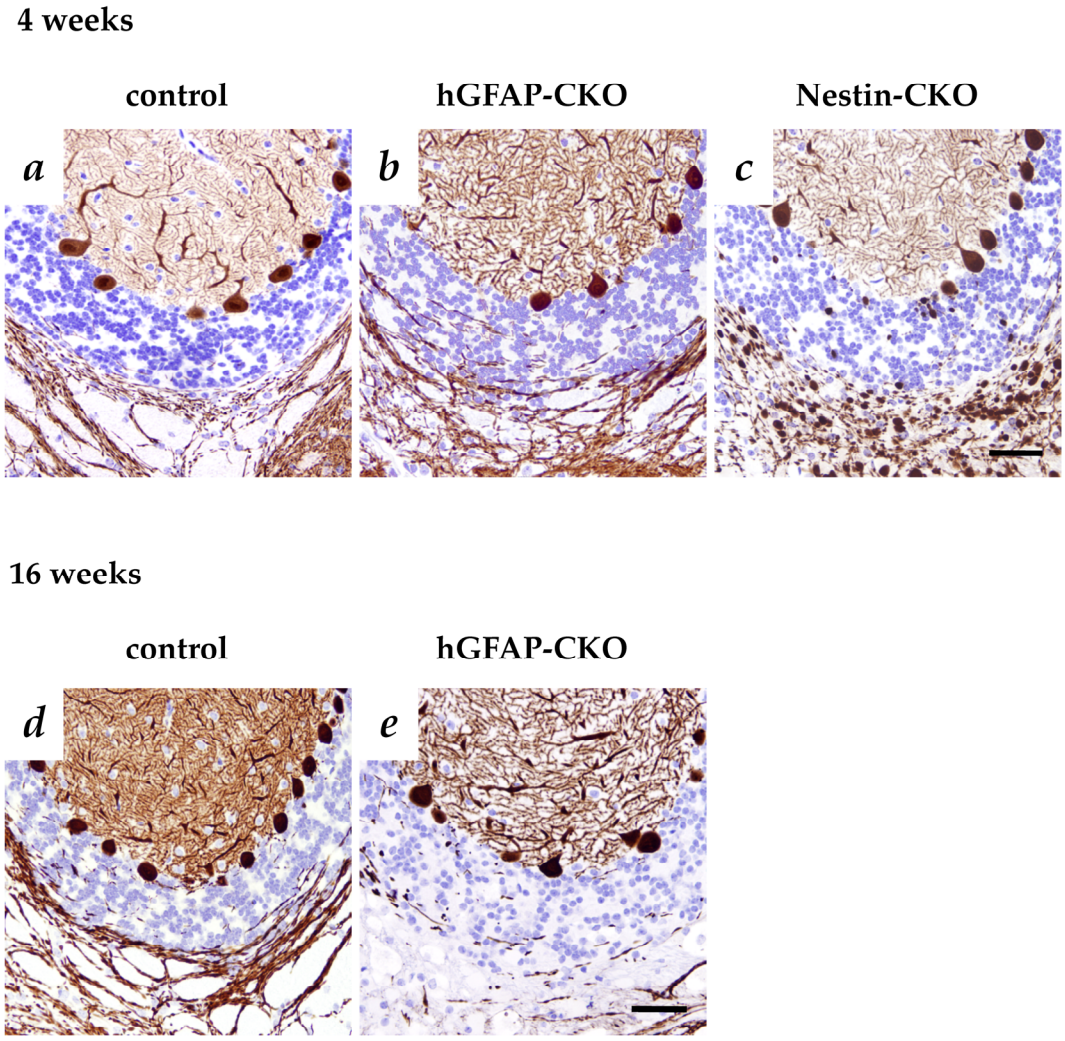


Figure 3.2 | Analysis of Purkinje cells in cerebellum of hGFAP-CKO mice. Cerebellar sections from control (*a*, *d*), hGFAP-CKO (*b*, *e*) and Nestin-CKO (*e*) mice at different ages were analyzed by immunohistochemistry with anti-calbindin. Scale Bars=100 μm.

Slight movement abnormality was observed for hGFAP-CKO mice compared to the littermate controls around 12 weeks of age and the phenotype of movement incoordination progressed to paralysis by about 20 weeks of age. Figure 3.3 shows the significant deficit in the motor coordination of hGFAP-CKO mice compared to the control mice at 16 weeks of age as measured by Rota-Rod tests. Consistent with the neurological defects at the older age, histological analysis of cerebellum sections showed a decreased number of Purkinje cells and a slightly reduced thickness of the molecular layer in hGFAP-CKO mice when compared to control mice (Figure 3.1, panels *d* and *e*). Furthermore, calbindin staining confirmed the reduction of Purkinje cell numbers and revealed significant degenerations of Purkinje cell axons in hGFAP-CKO mice when compared to control mice (Figure 3.2, panels *d* and *e*). Because hGFAP-Cre is not expressed in Purkinje cells, degeneration of Purkinje cell axons was likely caused by the defective granule cells in the older hGFAP-CKO mice as previous studies suggested that abnormalities in granule cells and/or glial cells could lead to degeneration of Purkinje cells (Baptista et al., 1994; Morrison and Mason, 1998). These results are also consistent with the late-onset of the ataxia phenotype in these mice compared to Nestin-CKO mice and provide additional support for the critical role of Purkinje cells in cerebellar degeneration and ataxia of the mutant mice upon deletion of FIP200 either directly (in Purkinje cells by nestin-Cre) or indirectly (in neurons other than Purkinje cells by hGFAP-Cre).

3.3.2 Ablation of FIP200 leads to cell-autonomous degeneration of the Purkinje cells and cerebellar ataxia

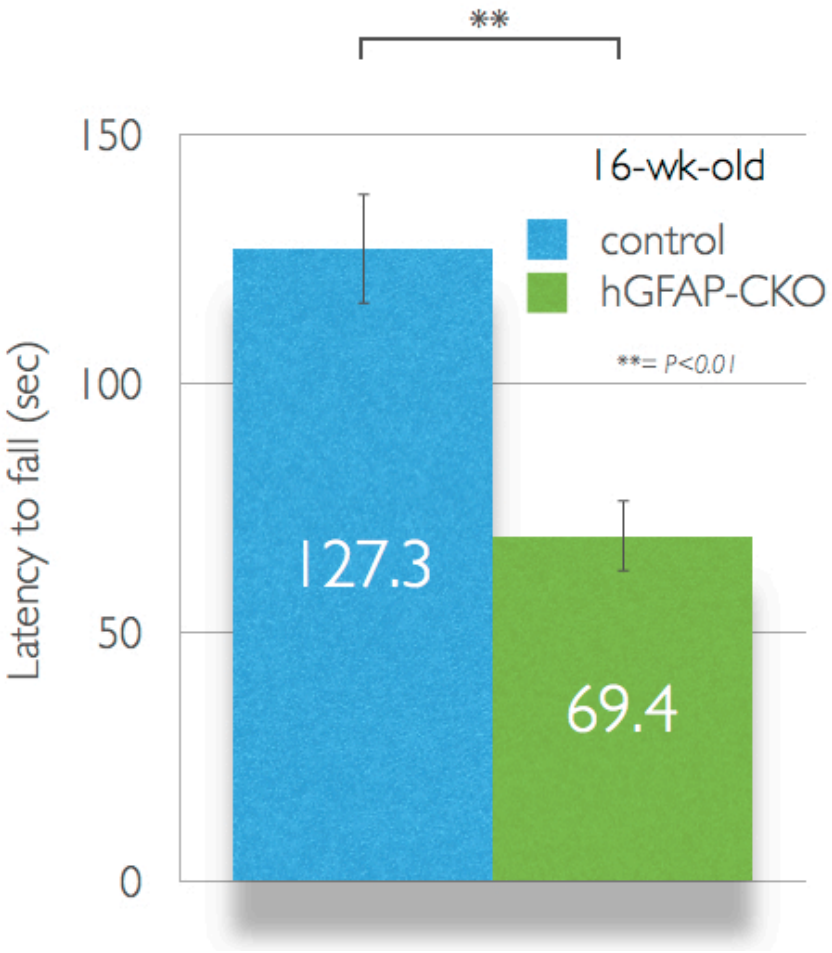


Figure 3.3 | Analysis of motor coordination in hGFAP-CKO mice. Control (n=4) or hGFAP-CKO (n=3) mice were placed on accelerating rotarods and latency to fall was recorded. The mean ± s.e. are shown.

To complement the above studies and further validate the Purkinje cell-autonomous defects in Nestin-CKO mice, I generated FIP200 conditional KO mice using L7-Cre mice which express Cre only in Purkinje cells (Lewis et al., 2004; Smeyne et al., 1995). As expected, the *FIP200*$^{flox/flox}$;L7-Cre (designated L7-CKO) mice were produced at Mendelian ratio and the mice were viable until at least 6 months of age. However, signs of ataxia were observed in the mutant mice by about 4 weeks of age. Measurement of motor coordination by Rota-Rod test showed a slight deficit in 4-week old mice, which progressed to a severer level by 8 weeks of age and beyond (Figure 3.4). I next examined the potential degeneration of Purkinje cells and their neurites by staining of the cerebellar sections with anti-calbindin (Figure 3.5). At 4 weeks of age, the number of Purkinje cells as well as their dendrites (Figure 3.5, panel *a*) and axons in the white matter (Figure 3.5, panel *b*) in the L7-CKO mice were comparable to those in the control mice (data not shown). However, calbindin staining revealed swelling segments of Purkinje cell axons (arrows), indicating their degeneration in L7-CKO mice. In addition, spongiform degeneration was observed in the white matter of L7-CKO mice (Figure 3.9, panel *c*). At 8 weeks of age, a significant loss of Purkinje cells and signs of degeneration of dendrites was observed in the mutant mice (Figure 3.5, panel *c*). Furthermore, more axonal swelling (arrows, Figure 3.5, panel *c*) and a reduced density of axons in the white matter (Figure 3.5, panel *d*) were found for the mutant mice compared to that at 4 weeks.

At 16 weeks of age, few Purkinje cells were left (Figure 3.5, panel *e*), a further degeneration of dendrites and axons as well as increased size of the axonal swelling (Figure 3.5, panels *e* and *f*) were observed for the mutant mice. Lastly, similar to Nestin-CKO mice, extensively abnormal ubiquitinated

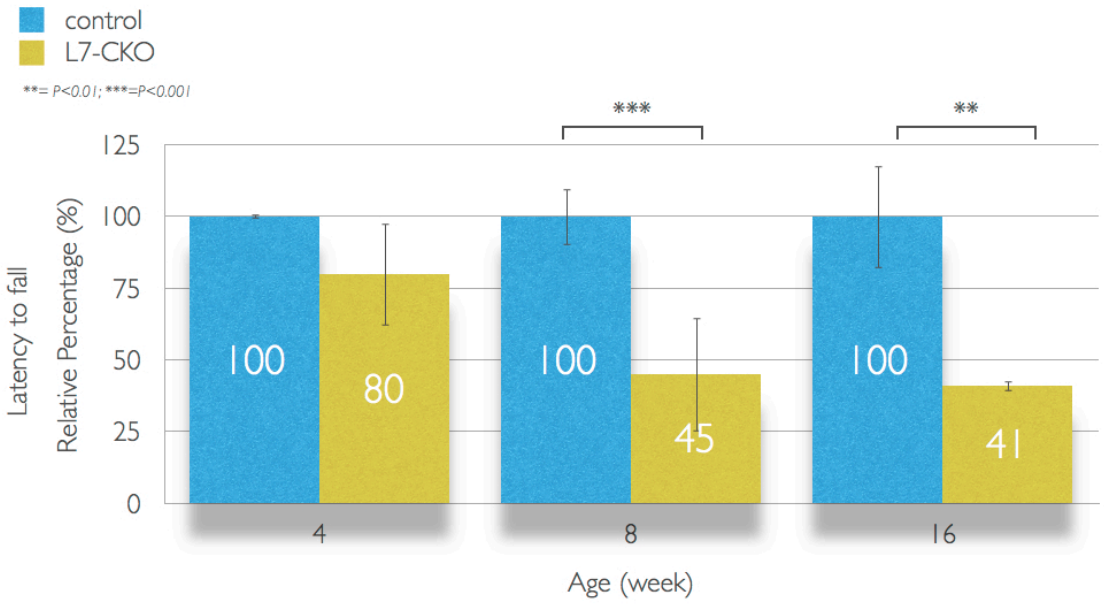
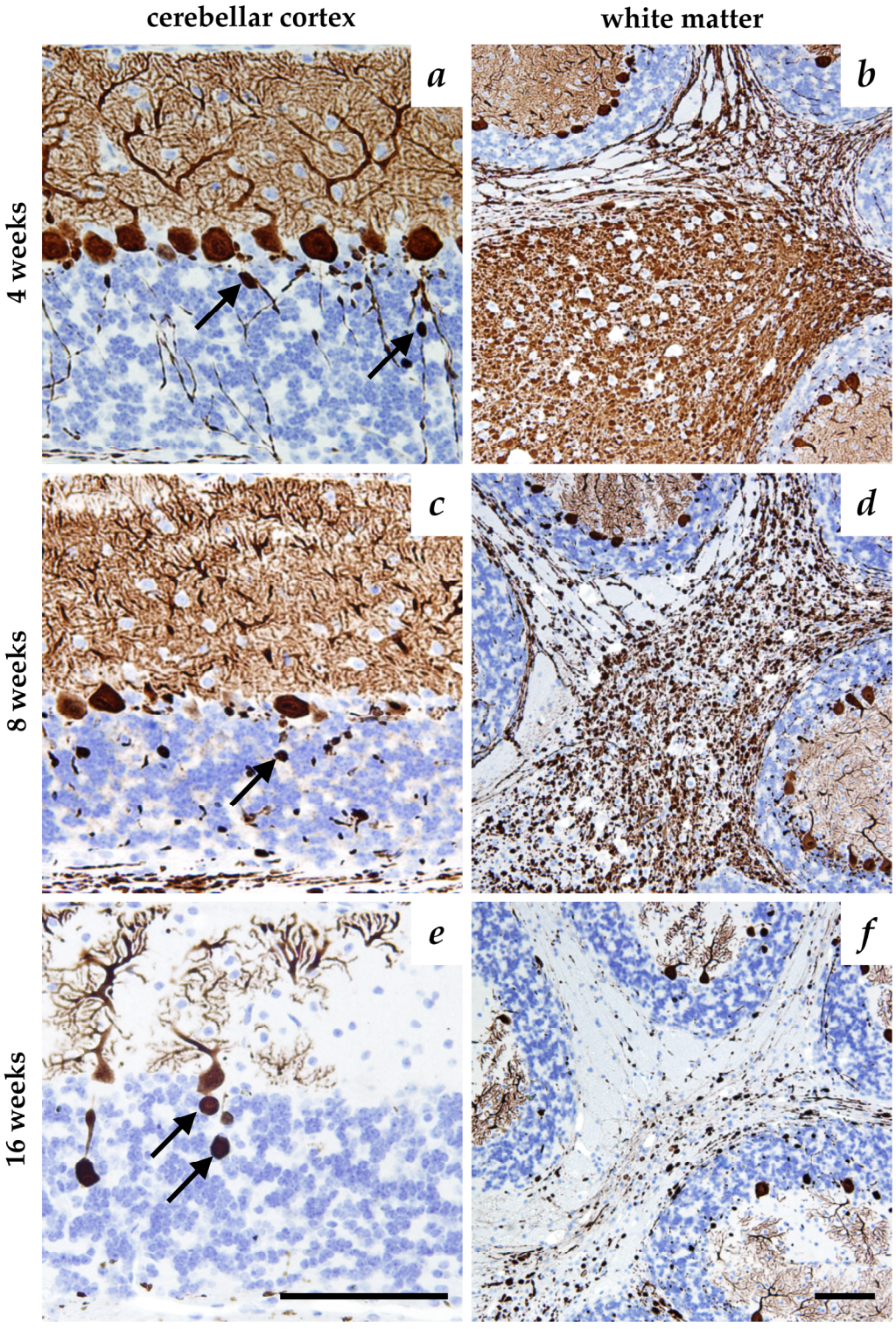


Figure 3.4 | Movement ataxia in L7-CKO mice. Control (n=5) or L7-CKO (n=5) mice at different ages were placed on accelerating rotarods and latency to fall were recorded. Note that data were normalized to control. The mean ± s.e. are shown.

Figure 3.5 | Degeneration of Purkinje cells in L7-CKO mice. Cerebellar sections from control L7-CKO mice at different ages were analyzed by immunohistochemistry with anti-calbindin. Note the progressive loss of Purkinje cells (*a, c, e*), their axons in white matter (*b, d, f*), and increasing swelling of the axons traversing the granule layer (arrows). Scale bars= 200μm.


cerebellar cortex
white matter
4 weeks
8 weeks
16 weeks
a
b
c
d
e
f

protein aggregates were also detected in the Purkinje cells and white matter of the L7-CKO mice (Figure 3.9, panel *e*). Together, these results strongly suggest that FIP200 plays a crucial role in the homeostasis of Purkinje cells in the cerebellum and that neural-specific deletion of FIP200 causes cerebellar degeneration and ataxia in a Purkinje cell-autonomous manner in mice.

3.3.3 Deletion of FIP200 leads to deficiency of autophagosome and abnormal mitochondria

To explore potential mechanisms by which deletion of FIP200 leads to neurodegeneration, I examined possible increases in apoptosis and ubiquitinated protein aggregates in the cerebellum of the mutant mice as many neurodegenerative diseases are associated with accumulation of ubiquitinated protein aggregates and loss of neurons resulting from apoptosis (Lossi and Merighi, 2003; Okouchi et al., 2007; Yuan et al., 2003). Owed to many neurodegenerative diseases resulted from the deficiency of protein quality control systems (UPS and autophagy), I first examined whether the proteasome activity is altered by deletion of FIP200 in the cerebellum, as impairment of proteasome functions could also lead to abnormal accumulation of ubiquitinated protein aggregates (Tai and Schuman, 2008). The caspase-like, chymotryptic and tryptic activities of the 20S proteosomes were measured by using Ac-GPLD-AMC (and Z-LLE-AMC), Suc-LLVYAMC, and Ac-RLR-AMC (and Boc-LRR-AMC) peptides, respectively, as substrates. I found that both the caspase-like and chymotryptic activities were elevated in the cerebella of Nestin-CKO mice compared to that from control mice at P14 and P28 (Figure 3.6). The tryptic activity was comparable for the mutant and control mice. The increased caspase-like and chymotryptic activities could be

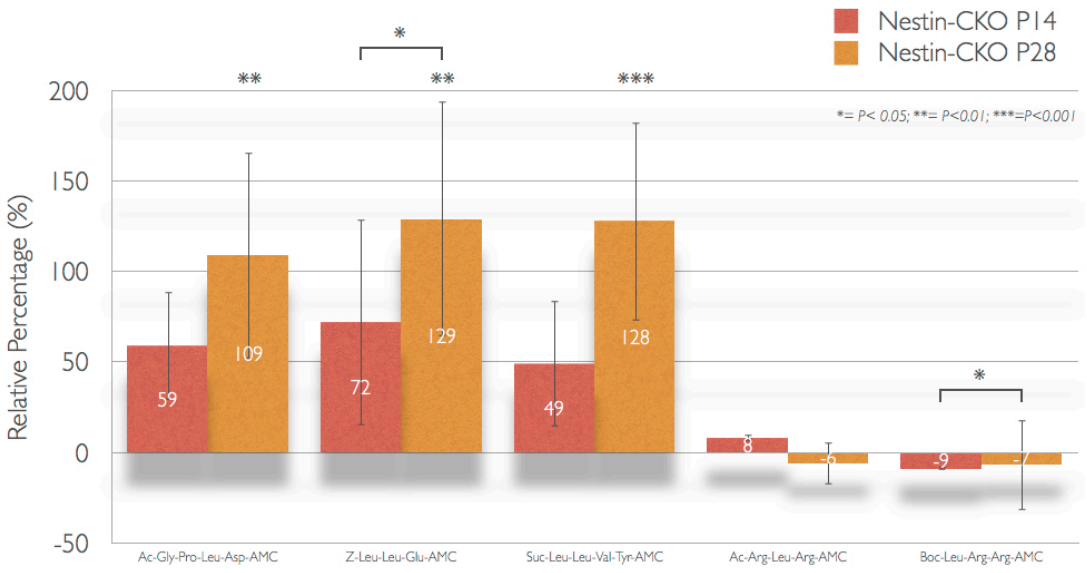
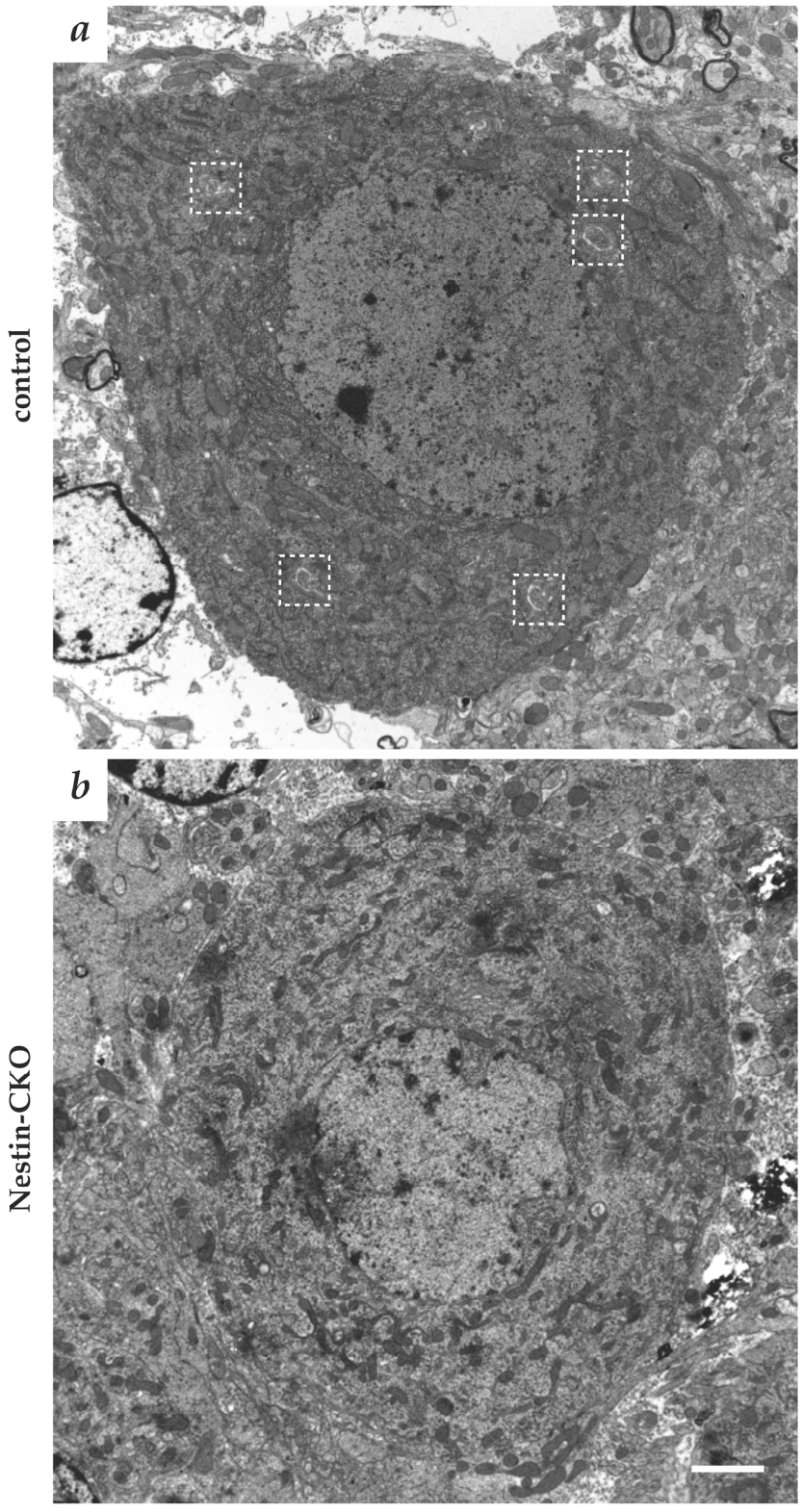


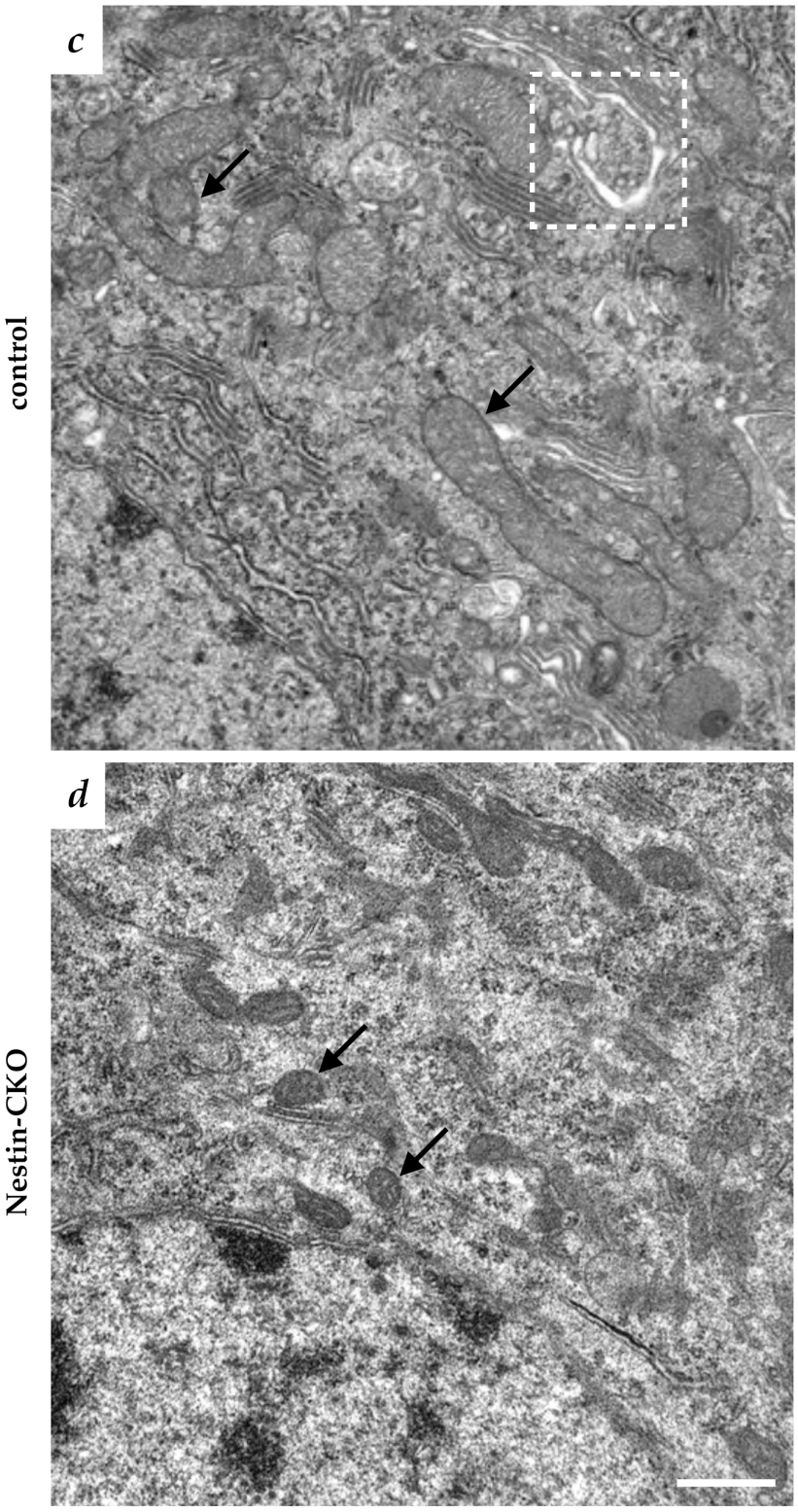
Figure 3.6 | Proteasome catalytic activity in cerebellum of Nestin-CKO mice. Lysates prepared from cerebellum of control or Nestin-CKO mice at P14 or P28 were measured for their proteasome activities using various substrates as indicated. Note that all data were normalized to controls as 100%. The mean ± s.e. from two independent experiments is shown.

resulting from some feedback mechanism for the cells to attempt to degrade the excess accumulation of the ubiquitinated protein aggregates. Together, these results suggested that the abnormal accumulation of ubiquitinated protein aggregates was likely a consequence of defective autophagy, but not decreased proteosome activity, in the CNS of mutant mice.

Recent studies have shown that basal autophagy is another important mechanism to remove abnormal protein aggregates (Hara et al., 2006; Komatsu et al., 2006). Suppression of basal autophagy by conditional KO of Atg5 or Atg7 in neuronal cells led to abnormal accumulation of ubiquitinated protein aggregates and increased apoptosis resulting in neurodegeneration in the mutant mice (Hara et al., 2006; Komatsu et al., 2006). Interestingly, FIP200 has been found recently to interact with ULK1 and 2, the mammalian orthologs of yeast Atg1, and the interaction is required for autophagosome formation in MEFs (Hara et al., 2008). To investigate potential deficiency in autophagosome formation that might account for the abnormal accumulation of ubiquitinated protein aggregates upon FIP200 deletion *in vivo*, I examined Purkinje cells in the cerebellum of Nestin-CKO and control mice by transmission electron microscopy. As shown in Figure 3.7, multiple autophagosomes characterized by the double membrane structures were identified in Purkinje cells from control mice (panels *a* and *c*, bracketed), but none were detected in those from Nestin-CKO (panels *b* and *d*). Furthermore, in contrast to the smooth and extended morphology of mitochondria in control Purkinje cells (panel *c*, arrows), mitochondria in the mutant Purkinje cells were smaller with a more condensed contents and sometimes fragmented morphology (panel *d*, arrows). These results suggested that defective autophagy upon deletion of FIP200 may be responsible for the accumulation

Figure 3.7 | Transmission electron microscopy of the Purkinje cells in Nestin-CKO mice. Purkinje cells from control (*a*, *c*) and Nestin-CKO (*b*, *d*) mice at 4 weeks of age were examined by transmission electron microscopy. Autophagosomes and mitochondria are marked by dotted rectangles and arrows, respectively. Scale bars= 2 μm (*a*, *b*) and 500 nm (*c*, *d*), respectively.


a
control
b
Nestin-CKO


c
control
d
Nestin-CKO

of ubiquitinated protein aggregates. Particularly, the accumulation of ubiquitinated protein aggregates in the Purkinje cells could be responsible for the loss of these cells and their axonal degeneration leading to cerebellar ataxia phenotype in the Nestin-CKO mice. Besides, the presence of abnormal mitochondria in the mutant cells could be a direct consequence of FIP200 deletion and owed to the deficiency in autophagy to remove the damaged mitochondria efficiently.

3.3.4 Deletion of FIP200 mediated loss of the Purkinje cells and spongiform degeneration is through TNFR-1 pathway

The above analysis raised the possibility that autophagy defects in Purkinje cells could lead to increased apoptosis resulting in the loss of Purkinje cells and spongiform degeneration upon FIP200 deletion. Yet, the increased apoptosis in the cerebellum of Nestin-Cre mice was primarily granule cells and other neurons in the white matter rather than Purkinje cells *per se* (Hara et al., 2006; Komatsu et al., 2006). Indeed, I could not detect any apoptotic Purkinje cells by TUNEL assay (data not shown) in L7-CKO mice despite evident progressive loss of these cells. Interestingly, a previous study suggested that the P75 neurotrophic receptor induces autophagy and Purkinje cell death (Florez-McClure et al., 2004). To assess the molecular mechanism of loss of Purkinje cells and spongiform degeneration upon FIP200 deletion *in vivo*, I introduced floxed *p53* allele into L7-CKO mice and generated *FIP200*$^{flox/flox}$;*p53*$^{flox/flox}$;L7-Cre double conditional KO mice because up-regulation of p53 has been indicated as a major mechanism for increased apoptosis in neuronal cells (Anderson and Tolkovsky, 1999; Jordan et al., 1997; Martin et al., 2005; Martin and Liu, 2002; Wong et al., 2005; Wood and Youle, 1995). Comparison

of the cerebellum sections of these mice and L7-CKO mice by calbindin staining showed similar extent of Purkinje cell loss (Figure 3.8, panels *a* and *b*). Furthermore, inactivation of p53 did not rescue spongiform degeneration (Figure 3.8, panel *c*) or accumulation of ubiquitinated protein aggregates (data not shown) in L7-CKO mice either. Together, these results suggest that Purkinje cell death upon FIP200 deletion is not through p53-mediated apoptosis.

Depletion of FIP200 has been shown to increase apoptosis in the embryonic heart and liver and analysis using MEFs and hepatocytes from FIP200 KO embryos suggested that increased sensitivity to TNFα-induced apoptosis may be responsible for the defective phenotype observed in FIP200 KO embryos (Gan et al., 2006). TNFα is expressed at a low level in the developing brain, which is required for the preservation of synaptic strength (Beattie et al., 2002; Dziegielewska et al., 2000; Munoz-Fernandez and Fresno, 1998). However, increased level of TNFα in neuronal disorders has been shown to cause damage to various neurons (Barone et al., 1997; Dawson et al., 1996). Therefore, it is possible that deletion of FIP200 in Purkinje cells will increase their sensitivity to TNFα (similar to MEFs), leading to decreased survival. To test such a possibility directly, L7-CKO mice were crossed with TNFR-1 KO mice to generate *FIP200*$^{flox/flox}$;L7-Cre;*TNFR-1*$^{-/-}$ double KO mice (designated TNFR1dL7-CKO). TNFR-1 is the major receptor for TNFα, and although they have some defects in the immune system, TNFR-1 KO mice do not exhibit any neurological disorders (Rothe et al., 1993). Figure 3.9 shows that deletion of TNFR-1 significantly reduced the Purkinje cell loss (compare panels *a* and *b*) eliminated spongiform degeneration (compare panels *c* and *d*) in cerebellum of L7-CKO mice. However, accumulation of ubiquitinated

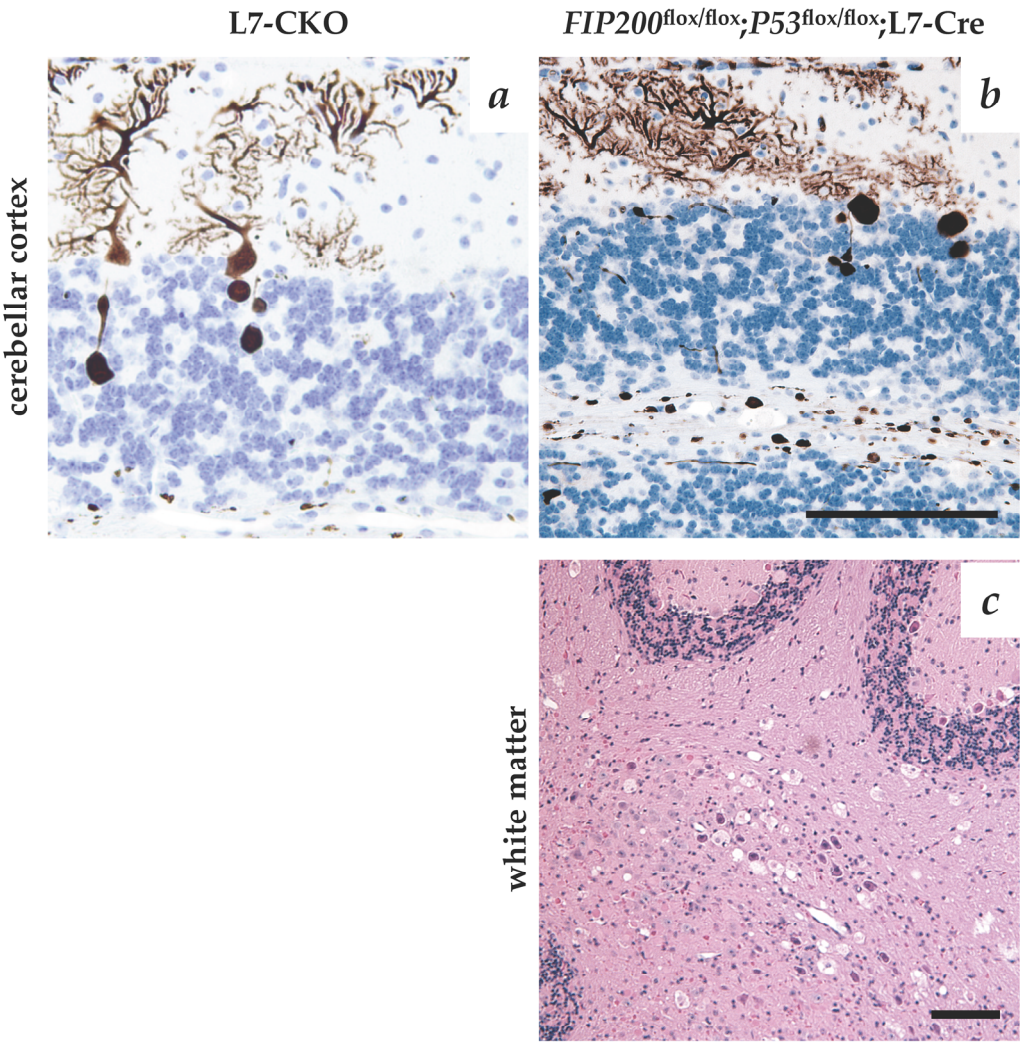
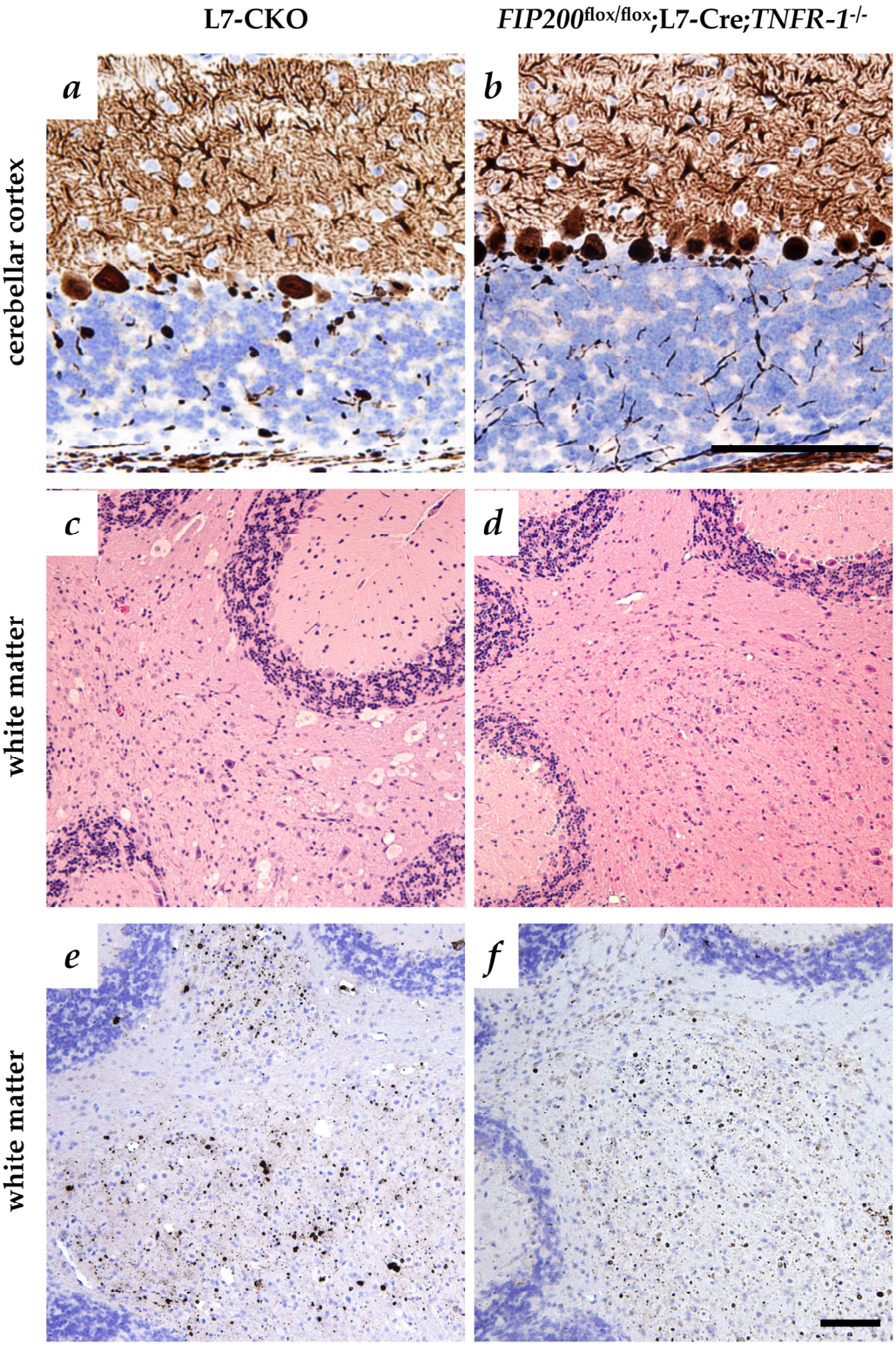


Figure 3.8 | Analysis of the Purkinje cells and spongiform degeneration in *FIP200*$^{flox/flox}$;*P53*$^{flox/flox}$;L7-Cre mice. Cerebellum sections from L7-CKO (*a*) or FIP200$^{flox/flox}$;p53$^{flox/flox}$;L7-Cre (*b*, *c*) at 16 weeks were analyzed by immunohistochemistry with anti-calbindin (*a*, *b*) or staining with H/E (*c*). Scale bars=200 μm.

Figure 3.9 | Analysis of the Purkinje cells and spongiform degeneration in *FIP200*$^{flox/flox}$;L7-Cre;*TNFR-1*$^{-/-}$ mice. Cerebellum sections from L7-CKO (*a*, *c*, *e*) or *FIP200*$^{flox/flox}$;L7-Cre;*TNFR-1*$^{-/-}$ (TNFR-1dL7-CKO)(*b*, *d*, *f*) at 8 weeks were analyzed by immunohistochemistry with anti-calbindin (*a*, *b*), anti-ubiquitin (*e*, *f*) or staining with H/E (*c*, *d*). Scale bars=200 μm.


L7-CKO
FIP200flox/flox;L7-Cre;TNFR-1-/-
a
b
cerebellar cortex
c
d
white matter
e
f
white matter

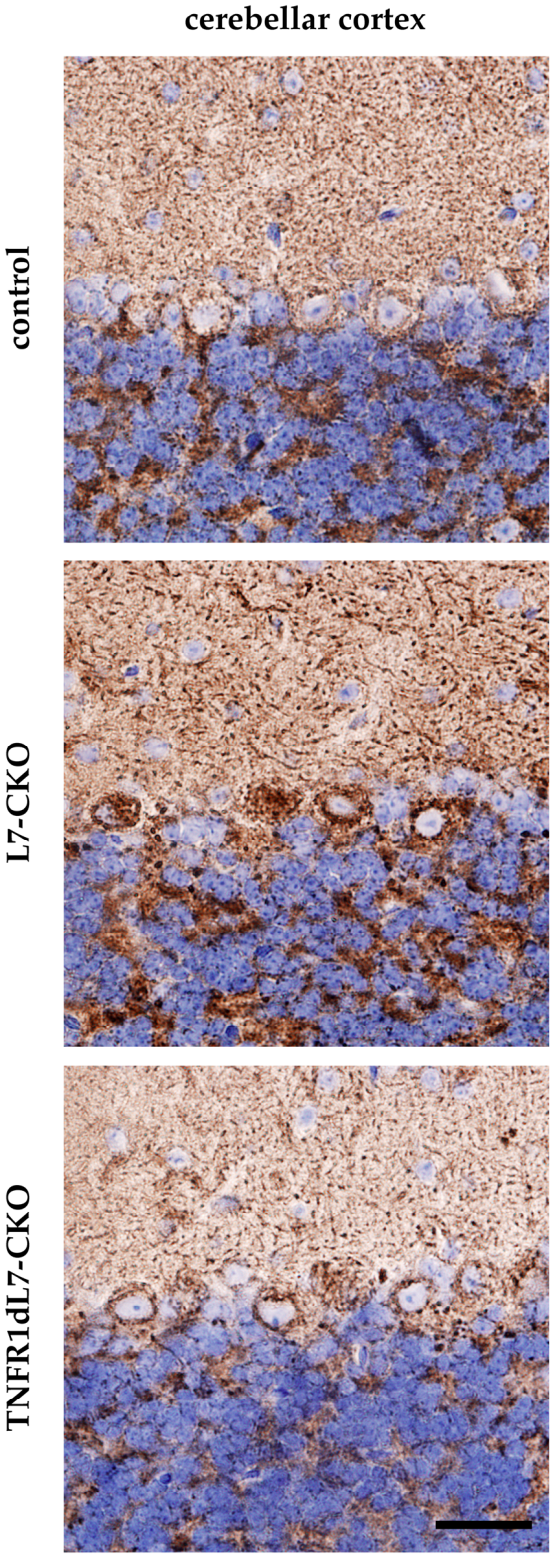
protein aggregates remained existing in both transgenic mice (see panels *e* and *f*).

In addition, it has been known that activation of TNFR-1 can trigger cell death by releasing cytochrome c from mitochondria (Guicciardi et al., 2000; Kim and Kim, 2003; Mabrouk et al., 2007). As shown in Figure 3.7, deletion of FIP200 led to an accumulation of abnormal mitochondria in the Purkinje cells. Figure 3.9 (compare panel *a* and *b*) also indicated that the depletion of TNFR-1 reduced loss of the Purkinje cells. Hence, an increased sensitivity to cell death in FIP200-depleted Purkinje cells may be resulting from TNFR-1-mediated cytochrome c release. To test the possibility, I next examined the Purkinje cells in L7-CKO and TNFR-1dL7-CKO by staining of the cerebellar sections with anti-cytochrome c (Figure 3.10). Interestingly, I found that a notable cytochrome c release in the Purkinje cells of L7-CKO mice (compare panel *a* and *b*). The cytochrome c release in the Purkinje cells of L7-CKO was significantly reduced by inactivation of TNFR-1. (compare panel *b* and *c*) Lastly, I found that TNFR-1 KO partially restored the motor coordination of L7-CKO mice as measured by Rota-Rod tests (Figure 3.11). These results suggest that increased sensitivity to TNFα-induced cell death may at least partly account for Purkinje cell loss and associated spongiform degeneration upon deletion of FIP200.

3.4 Discussion

Purkinje cells are the master neurons of the cerebellar cortex and are the only efferent relay to the central nervous system through their axon connections to the deep cerebellar nuclei. These cells are born during

Figure 3.10 | Inactivation of TNFR1 reduce cytochrome c release in the Purkinje cells of L7-CKO. Cerebellum sections from control, L7-CKO or TNFR-1dL7-CKO at 8 weeks were analyzed by immunohistochemistry with anti-cytochrome c. Scale bars=50 μm.


cerebellar cortex
control
L7-CKO
TNFR1dL7-CKO

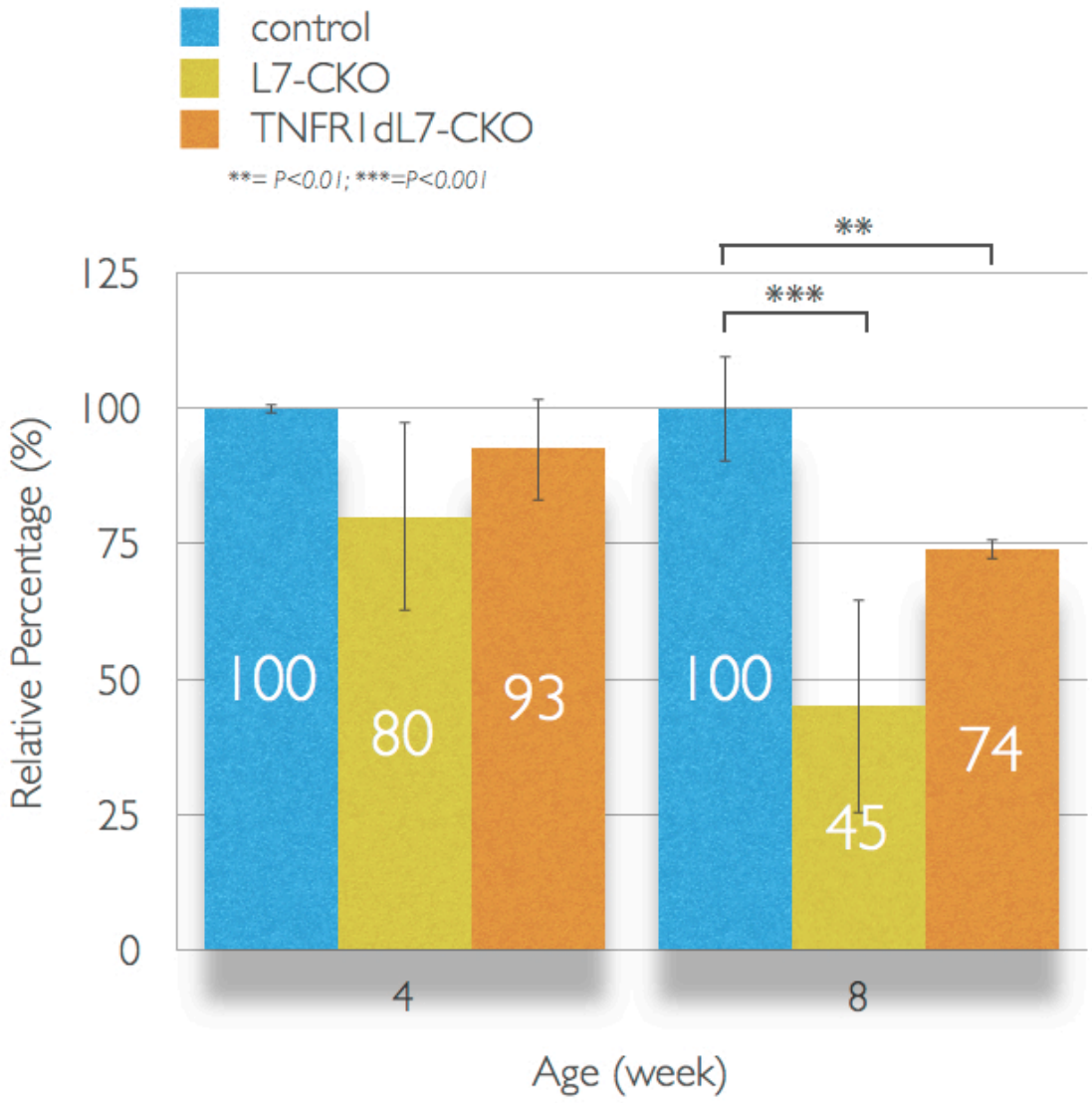


Figure 3.11 | Movement ataxia in TNFR1dL7-CKO mice. Control, L7-CKO or TNFR1dL7-CKO (n=3) mice at different ages were placed on accelerating rotarods and latency to fall were recorded. Note that data were normalized to control. The mean ± s.e. are shown.

embryogenesis (Leclerc et al., 1988; Oberdick et al., 1993; Seil et al., 1995; Wassef et al., 1990) and form a monolayer separating the molecular and the internal granule layers by the first 2-3 postnatal weeks (Altman, 1972a; Altman, 1972b; Goldowitz and Hamre, 1998). My studies using three different Cre lines with complementary patterns of Cre expression in the cerebellum provided strong support for a Purkinje cell-autonomous function of FIP200 in the homeostasis of these cells and cerebellar ataxia upon its inactivation. Mice with deletion of FIP200 mediated by nestin-Cre or L7-Cre both developed cerebellar ataxia within a few weeks of age. Deletion of FIP200 mediated by hGFAP-Cre, which does not express Cre in Purkinje cells, did not show any neurological disorders within the same time-frame. The later appearance of ataxia also correlated to the loss of Purkinje in these mice, which likely results from the deficiency in granule cells or other neurons in the cerebellum. My studies identified a function for FIP200 in the regulation of neuronal cell homeostasis and its inactivation caused cerebellar degeneration and ataxia accompanied with progressive accumulation of abnormal ubiquitinated protein aggregates, loss of Purkinje cells, axonal swelling and spongiform degeneration.

Protein quality control mechanisms including the UPS and autophagy have been suggested to play crucial roles in the homeostasis of neurons and in neurodegeneration (Berke and Paulson, 2003; Ciechanover, 2006; Mizushima and Klionsky, 2007; Pickart, 2004; Rubinsztein, 2006). Nevertheless, the molecular components and signaling pathways that regulate these cellular processes in various neurodegenerative diseases are still not well understood at present. Deletion of FIP200 resulted in neurological defects that share several characteristics with the recently reported Atg5 or Atg7 conditional KO

mice by nestin-Cre (Hara et al., 2006; Komatsu et al., 2006), including the increased ubiquitin aggregates without any deficiency in proteasome catalytic functions. Interestingly, a recent study identified an interaction between FIP200 and ULK1, the mammalian homolog of yeast Atg1, and showed that FIP200 is required for starvation-induced autophagy in mammalian cells *in vitro* (Hara et al., 2008). Thus, the data presented here strongly suggest a role of FIP200 in the regulation of autophagy *in vivo* and deficiency in autophagy upon FIP200 inactivation is likely a major contributor to neurodegeneration in the mutant mice. The contents of the abnormally accumulated ubiquitinated protein aggregates in the FIP200 conditional KO mice, nor those in the Atg5 or Atg7 conditional KO mice are unknown. However, it is conceivable that different protein aggregates were accumulated in the FIP200 conditional KO mice vs Atg5 or Atg7 conditional KO mouse, which may account for the differential phenotype in FIP200 conditional KO mice. In some cases of neurodegenerative mice KO models, the responsible protein aggregates contributing to the neuronal swelling and neurodegeneration are known, such as Prion in TSE (Hooper, 2003) and TDP-43 in *Sod1*$^{-/-}$ (Arai et al., 2006; Neumann et al., 2006). Future studies will be directed at identifying the proteins in the ubiquitinated aggregates of FIP200 conditional KO mice and determining whether any of these are responsible for the axonal swelling, spongiform degeneration, and other cerebellar degenerative phenotypes.

My studies also characterized several distinctive neurological features of neural-specific FIP200 conditional KO mice including the progressive development of spongiform degeneration that were not observed in the Atg5 or Atg7 conditional KO mice (Hara et al., 2006; Komatsu et al., 2006) and indeed rarely found in other KO mice models (Akassoglou et al., 2004; Li et al.,

1995; Lin et al., 2004; Matalon et al., 2000; Puccio et al., 2001). Mitochondrial damage was also observed in FIP200 conditional KO mice, but not reported for Atg5 or Atg7 KO mice. FIP200 has been shown to regulate several other signaling pathways and cellular processes besides its interaction with ULK1 and regulation of autophagy (Hara et al., 2008). It is therefore possible that perturbations of other signaling pathways may contribute to these distinct phenotypes in the FIP200 conditional KO mice.

While my data excluded a role of p53-dependent apoptosis, I found that deletion of TNFR-1 rescued both the loss of Purkinje cells and spongiform degeneration in FIP200 conditional KO mice, suggesting that altered TNFα signaling may play a crucial role in cerebellar degeneration upon FIP200 deletion. Increased TNFα production from microglia associated with pro-inflammatory responses have been found in brain injury and neurodegeneration such as Alzheimer's (Bruunsgaard et al., 1999; Collins et al., 2000) and Parkinson's diseases (Boka et al., 1994; Dobbs et al., 1999; Mogi et al., 1994; Nishimura et al., 2001). However, I did not detect any signs of inflammation in the cerebellum of FIP200 conditional KO mice (data not shown). Thus, it is possible, or even likely, that deletion of FIP200 resulted in an increased sensitivity of Purkinje cells to TNFα-induced cell death, similar to our previous findings in FIP200 KO MEFs (Gan et al., 2006). It is also interesting to note that TNFR-1 deficiency only partially reduced accumulation of ubiquitinated protein aggregates, suggesting that altered TNFR-1 signaling is distinct from the autophagy defects caused by FIP200 inactivation. On the other hand, I also observed increased cytochrome c release in the Purkinje cells of L7-CKO was rescued by TNFR-1 deficiency. The observation suggests that TNFα signaling may play a crucial role in

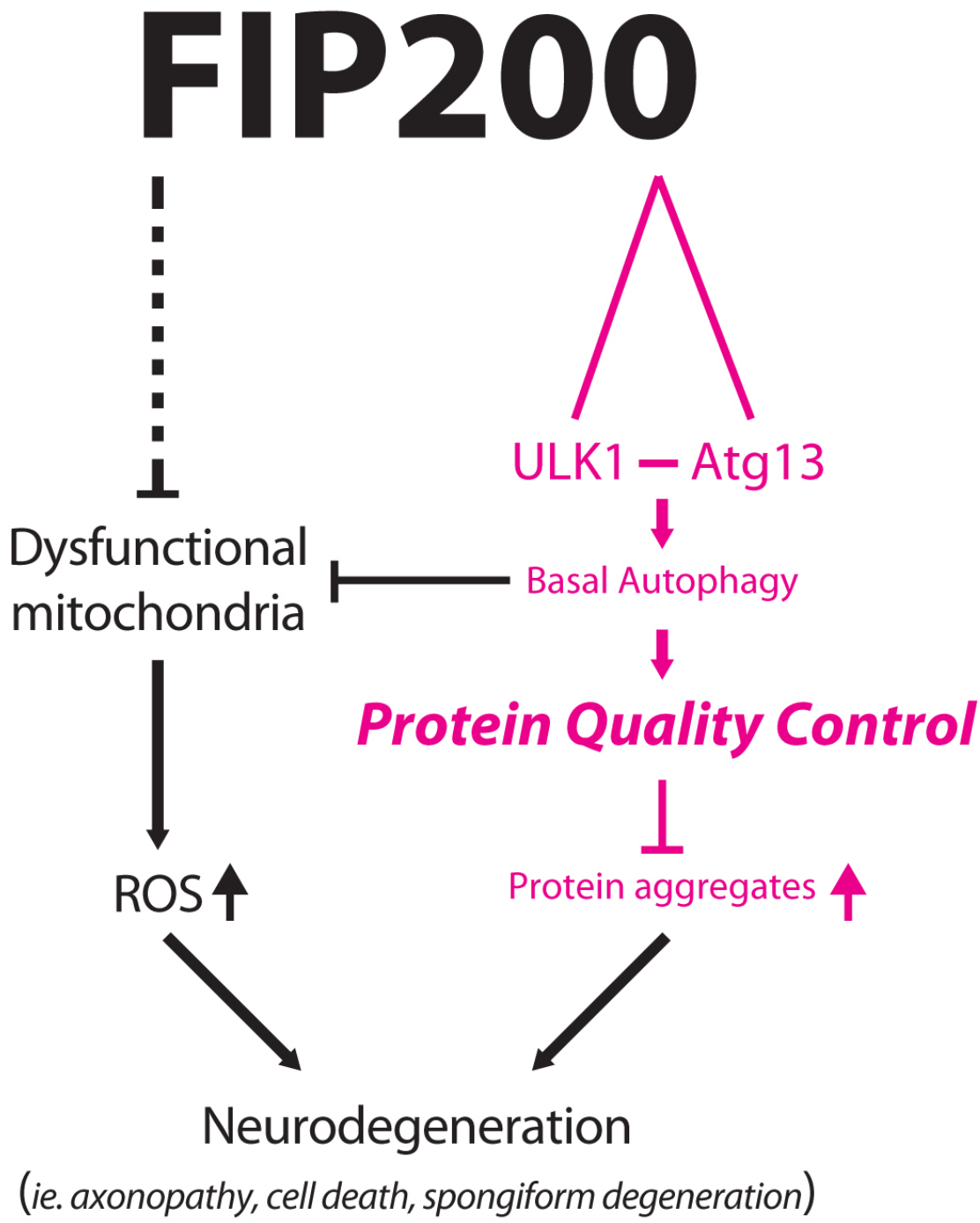


Figure 3.12 | Role of FIP200 in neurodegeneration.

mitochondrial damage independent of defective autophagy in these neurons while both could contribute to the loss of Purkinje cells and spongiform degeneration leading to cerebellar ataxia.

In summary, these results identified that FIP200 as a novel regulator of neuronal homeostasis and provide novel insight into molecular and cellular mechanisms of pathogenesis of neurodegenerative diseases as summarized in a working model (Figure 3.12). The set of FIP200 conditional KO mice may provide useful mice models for future studies on the mechanisms of cerebellar degeneration and ataxia as well as promising therapeutic approaches for the diseases in humans.

REFERENCES

Akassoglou, K., B. Malester, J. Xu, L. Tessarollo, J. Rosenbluth, and M.V. Chao. 2004. Brain-specific deletion of neuropathy target esterase/swisscheese results in neurodegeneration. *Proc Natl Acad Sci U S A*. 101:5075-80.

Altman, J. 1972a. Postnatal development of the cerebellar cortex in the rat. 3. Maturation of the components of the granular layer. *J Comp Neurol*. 145:465-513.

Altman, J. 1972b. Postnatal development of the cerebellar cortex in the rat. I. The external germinal layer and the transitional molecular layer. *J Comp Neurol*. 145:353-97.

Anderson, C.N., and A.M. Tolkovsky. 1999. A role for MAPK/ERK in sympathetic neuron survival: protection against a p53-dependent, JNK-independent induction of apoptosis by cytosine arabinoside. *J Neurosci*. 19:664-73.

Arai, T., M. Hasegawa, H. Akiyama, K. Ikeda, T. Nonaka, H. Mori, D. Mann, K. Tsuchiya, M. Yoshida, Y. Hashizume, and T. Oda. 2006. TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Biochem Biophys Res Commun*. 351:602-11.

Baptista, C.A., M.E. Hatten, R. Blazeski, and C.A. Mason. 1994. Cell-cell interactions influence survival and differentiation of purified Purkinje cells in vitro. *Neuron*. 12:243-60.

Barone, F.C., B. Arvin, R.F. White, A. Miller, C.L. Webb, R.N. Willette, P.G. Lysko, and G.Z. Feuerstein. 1997. Tumor necrosis factor-alpha. A mediator of focal ischemic brain injury. *Stroke*. 28:1233-44.

Bates, B., M. Rios, A. Trumpp, C. Chen, G. Fan, J.M. Bishop, and R. Jaenisch. 1999. Neurotrophin-3 is required for proper cerebellar development. *Nat Neurosci*. 2:115-7.

Beattie, E.C., D. Stellwagen, W. Morishita, J.C. Bresnahan, B.K. Ha, M. Von Zastrow, M.S. Beattie, and R.C. Malenka. 2002. Control of synaptic strength by glial TNFalpha. *Science*. 295:2282-5.

Berke, S.J., and H.L. Paulson. 2003. Protein aggregation and the ubiquitin proteasome pathway: gaining the UPPer hand on neurodegeneration. *Curr Opin Genet Dev*. 13:253-61.

Boka, G., P. Anglade, D. Wallach, F. Javoy-Agid, Y. Agid, and E.C. Hirsch. 1994. Immunocytochemical analysis of tumor necrosis factor and its receptors in Parkinson's disease. *Neurosci Lett*. 172:151-4.

Bruunsgaard, H., K. Andersen-Ranberg, B. Jeune, A.N. Pedersen, P. Skinhoj, and B.K. Pedersen. 1999. A high plasma concentration of TNF-alpha is associated with dementia in centenarians. *J Gerontol A Biol Sci Med Sci*. 54:M357-64.

Chano, T., S. Ikegawa, F. Saito-Ohara, J. Inazawa, A. Mabuchi, Y. Saeki, and H. Okabe. 2002. Isolation, characterization and mapping of the mouse and human RB1CC1 genes. *Gene*. 291:29-34.

Chano, T., H. Okabe, and C.M. Hulette. 2007. RB1CC1 insufficiency causes neuronal atrophy through mTOR signaling alteration and involved in the pathology of Alzheimer's diseases. *Brain Res*. 1168:97-105.

Chano, T., M. Saji, H. Inoue, K. Minami, T. Kobayashi, O. Hino, and H. Okabe. 2006. Neuromuscular abundance of RB1CC1 contributes to the non-proliferating enlarged cell phenotype through both RB1 maintenance and TSC1 degradation. *Int J Mol Med*. 18:425-32.

Ciechanover, A. 2006. Intracellular protein degradation: from a vague idea thru the lysosome and the ubiquitin-proteasome system and onto human diseases and drug targeting. *Exp Biol Med (Maywood)*. 231:1197-211.

Collins, J.S., R.T. Perry, B. Watson, Jr., L.E. Harrell, R.T. Acton, D. Blacker, M.S. Albert, R.E. Tanzi, S.S. Bassett, M.G. McInnis, R.D. Campbell, and R.C. Go. 2000. Association of a haplotype for tumor necrosis factor in siblings with late-onset Alzheimer disease: the NIMH Alzheimer Disease Genetics Initiative. *Am J Med Genet*. 96:823-30.

Cuervo, A.M. 2004. Autophagy: in sickness and in health. *Trends Cell Biol*. 14:70-7.

Dahlstrand, J., M. Lardelli, and U. Lendahl. 1995. Nestin mRNA expression correlates with the central nervous system progenitor cell state in many, but not all, regions of developing central nervous system. *Brain Res Dev Brain Res*. 84:109-29.

Dawson, D.A., D. Martin, and J.M. Hallenbeck. 1996. Inhibition of tumor necrosis factor-alpha reduces focal cerebral ischemic injury in the spontaneously hypertensive rat. *Neurosci Lett*. 218:41-4.

Dobbs, R.J., A. Charlett, A.G. Purkiss, S.M. Dobbs, C. Weller, and D.W. Peterson. 1999. Association of circulating TNF-alpha and IL-6 with ageing and parkinsonism. *Acta Neurol Scand*. 100:34-41.

Dziegielewska, K.M., J.E. Moller, A.M. Potter, J. Ek, M.A. Lane, and N.R. Saunders. 2000. Acute-phase cytokines IL-1beta and TNF-alpha in brain development. *Cell Tissue Res*. 299:335-45.

Florez-McClure, M.L., D.A. Linseman, C.T. Chu, P.A. Barker, R.J. Bouchard, S.S. Le, T.A. Laessig, and K.A. Heidenreich. 2004. The p75 neurotrophin

receptor can induce autophagy and death of cerebellar Purkinje neurons. *J Neurosci*. 24:4498-509.

Frappart, P.O., Y. Lee, J. Lamont, and P.J. McKinnon. 2007. BRCA2 is required for neurogenesis and suppression of medulloblastoma. *EMBO J*. 26:2732-42.

Gan, B., and J.L. Guan. 2008. FIP200, a key signaling node to coordinately regulate various cellular processes. *Cell Signal*. 20:787-94.

Gan, B., X. Peng, T. Nagy, A. Alcaraz, H. Gu, and J.L. Guan. 2006. Role of FIP200 in cardiac and liver development and its regulation of TNFalpha and TSC-mTOR signaling pathways. *J Cell Biol*. 175:121-33.

Ganley, I.G., D.H. Lam, J. Wang, X. Ding, S. Chen, and X. Jiang. 2009. ULK1-ATG13-FIP200 complex mediates mTOR signaling and is essential for autophagy. *J Biol Chem*.

Goldowitz, D., and K. Hamre. 1998. The cells and molecules that make a cerebellum. *Trends Neurosci*. 21:375-82.

Guicciardi, M.E., J. Deussing, H. Miyoshi, S.F. Bronk, P.A. Svingen, C. Peters, S.H. Kaufmann, and G.J. Gores. 2000. Cathepsin B contributes to TNF-alpha-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome c. *J Clin Invest*. 106:1127-37.

Hara, T., and N. Mizushima. 2009. Role of ULK-FIP200 complex in mammalian autophagy: FIP200, a counterpart of yeast Atg17? *Autophagy*. 5:85-7.

Hara, T., K. Nakamura, M. Matsui, A. Yamamoto, Y. Nakahara, R. Suzuki-Migishima, M. Yokoyama, K. Mishima, I. Saito, H. Okano, and N. Mizushima. 2006. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature*. 441:885-9.

Hara, T., A. Takamura, C. Kishi, S. Iemura, T. Natsume, J.L. Guan, and N. Mizushima. 2008. FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells. *J Cell Biol*. 181:497-510.

Hippert, M.M., P.S. O'Toole, and A. Thorburn. 2006. Autophagy in cancer: good, bad, or both? *Cancer Res*. 66:9349-51.

Hooper, N.M. 2003. Could inhibition of the proteasome cause mad cow disease? *Trends Biotechnol*. 21:144-5.

Hosokawa, N., T. Hara, T. Kaizuka, C. Kishi, A. Takamura, Y. Miura, S.I. Iemura, T. Natsume, K. Takehana, N. Yamada, J.L. Guan, N. Oshiro, and N. Mizushima. 2009. Nutrient-dependent mTORC1 Association with the ULK1-Atg13-FIP200 Complex Required for Autophagy. *Mol Biol Cell*.

Jonkers, J., R. Meuwissen, H. van der Gulden, H. Peterse, M. van der Valk, and A. Berns. 2001. Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. *Nat Genet*. 29:418-25.

Jordan, J., M.F. Galindo, J.H. Prehn, R.R. Weichselbaum, M. Beckett, G.D. Ghadge, R.P. Roos, J.M. Leiden, and R.J. Miller. 1997. p53 expression induces apoptosis in hippocampal pyramidal neuron cultures. *J Neurosci*. 17:1397-405.

Jung, C.H., C.B. Jun, S.H. Ro, Y.M. Kim, N.M. Otto, J. Cao, M. Kundu, and D.H. Kim. 2009. ULK-Atg13-FIP200 Complexes Mediate mTOR Signaling to the Autophagy Machinery. *Mol Biol Cell*.

Kim, H.H., and K. Kim. 2003. Enhancement of TNF-alpha-mediated cell death in vascular smooth muscle cells through cytochrome c-independent pathway by the proteasome inhibitor. *FEBS Lett*. 535:190-4.

Klionsky, D.J. 2005. The molecular machinery of autophagy: unanswered questions. *J Cell Sci*. 118:7-18.

Komatsu, M., S. Waguri, T. Chiba, S. Murata, J. Iwata, I. Tanida, T. Ueno, M. Koike, Y. Uchiyama, E. Kominami, and K. Tanaka. 2006. Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature*. 441:880-4.

Leclerc, N., C. Gravel, and R. Hawkes. 1988. Development of parasagittal zonation in the rat cerebellar cortex: MabQ113 antigenic bands are created postnatally by the suppression of antigen expression in a subset of Purkinje cells. *J Comp Neurol*. 273:399-420.

Levine, B., and D.J. Klionsky. 2004. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell*. 6:463-77.

Lewis, P.M., A. Gritli-Linde, R. Smeyne, A. Kottmann, and A.P. McMahon. 2004. Sonic hedgehog signaling is required for expansion of granule neuron precursors and patterning of the mouse cerebellum. *Dev Biol*. 270:393-410.

Li, Y., T.T. Huang, E.J. Carlson, S. Melov, P.C. Ursell, J.L. Olson, L.J. Noble, M.P. Yoshimura, C. Berger, P.H. Chan, D.C. Wallace, and C.J. Epstein. 1995. Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet*. 11:376-81.

Lin, J., P.H. Wu, P.T. Tarr, K.S. Lindenberg, J. St-Pierre, C.Y. Zhang, V.K. Mootha, S. Jager, C.R. Vianna, R.M. Reznick, L. Cui, M. Manieri, M.X. Donovan, Z. Wu, M.P. Cooper, M.C. Fan, L.M. Rohas, A.M. Zavacki, S. Cinti, G.I. Shulman, B.B. Lowell, D. Krainc, and B.M. Spiegelman. 2004. Defects in adaptive energy metabolism with CNS-linked hyperactivity in PGC-1alpha null mice. *Cell*. 119:121-35.

Lossi, L., and A. Merighi. 2003. In vivo cellular and molecular mechanisms of neuronal apoptosis in the mammalian CNS. *Prog Neurobiol*. 69:287-312.

Mabrouk, G.M., E.M. Ali, M.A. El-Rehany, and H.M. El-Samoly. 2007. TGF-beta1, TNF-alpha and cytochrome c in human astrocytic tumors: a short-term follow up and correlation with survival. *Clin Biochem*. 40:255-60.

Martin, L.J., K. Chen, and Z. Liu. 2005. Adult motor neuron apoptosis is mediated by nitric oxide and Fas death receptor linked by DNA damage and p53 activation. *J Neurosci*. 25:6449-59.

Martin, L.J., and Z. Liu. 2002. Injury-induced spinal motor neuron apoptosis is preceded by DNA single-strand breaks and is p53- and Bax-dependent. *J Neurobiol*. 50:181-97.

Matalon, R., P.L. Rady, K.A. Platt, H.B. Skinner, M.J. Quast, G.A. Campbell, K. Matalon, J.D. Ceci, S.K. Tyring, M. Nehls, S. Surendran, J. Wei, E.L. Ezell, and S. Szucs. 2000. Knock-out mouse for Canavan disease: a model for gene transfer to the central nervous system. *J Gene Med*. 2:165-75.

McKinney, B.C., and G.G. Murphy. 2006. The L-Type voltage-gated calcium channel Cav1.3 mediates consolidation, but not extinction, of contextually conditioned fear in mice. *Learn Mem*. 13:584-9.

Mizushima, N. 2007. Autophagy: process and function. *Genes Dev*. 21:2861-73.

Mizushima, N., and D.J. Klionsky. 2007. Protein turnover via autophagy: implications for metabolism. *Annu Rev Nutr*. 27:19-40.

Mizushima, N., B. Levine, A.M. Cuervo, and D.J. Klionsky. 2008. Autophagy fights disease through cellular self-digestion. *Nature*. 451:1069-75.

Mogi, M., M. Harada, P. Riederer, H. Narabayashi, K. Fujita, and T. Nagatsu. 1994. Tumor necrosis factor-alpha (TNF-alpha) increases both in the brain and in the cerebrospinal fluid from parkinsonian patients. *Neurosci Lett*. 165:208-10.

Morrison, M.E., and C.A. Mason. 1998. Granule neuron regulation of Purkinje cell development: striking a balance between neurotrophin and glutamate signaling. *J Neurosci*. 18:3563-73.

Munoz-Fernandez, M.A., and M. Fresno. 1998. The role of tumour necrosis factor, interleukin 6, interferon-gamma and inducible nitric oxide synthase in the development and pathology of the nervous system. *Prog Neurobiol*. 56:307-40.

Neumann, M., D.M. Sampathu, L.K. Kwong, A.C. Truax, M.C. Micsenyi, T.T. Chou, J. Bruce, T. Schuck, M. Grossman, C.M. Clark, L.F. McCluskey, B.L. Miller, E. Masliah, I.R. Mackenzie, H. Feldman, W. Feiden, H.A. Kretzschmar, J.Q. Trojanowski, and V.M. Lee. 2006. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science*. 314:130-3.

Nishimura, M., I. Mizuta, E. Mizuta, S. Yamasaki, M. Ohta, R. Kaji, and S. Kuno. 2001. Tumor necrosis factor gene polymorphisms in patients with sporadic Parkinson's disease. *Neurosci Lett*. 311:1-4.

Oberdick, J., K. Schilling, R.J. Smeyne, J.G. Corbin, C. Bocchiaro, and J.I. Morgan. 1993. Control of segment-like patterns of gene expression in the mouse cerebellum. *Neuron*. 10:1007-18.

Okouchi, M., O. Ekshyyan, M. Maracine, and T.Y. Aw. 2007. Neuronal apoptosis in neurodegeneration. *Antioxid Redox Signal*. 9:1059-96.

Pfeffer, K., T. Matsuyama, T.M. Kundig, A. Wakeham, K. Kishihara, A. Shahinian, K. Wiegmann, P.S. Ohashi, M. Kronke, and T.W. Mak. 1993. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. *Cell*. 73:457-67.

Pickart, C.M. 2004. Back to the future with ubiquitin. *Cell*. 116:181-90.

Puccio, H., D. Simon, M. Cossee, P. Criqui-Filipe, F. Tiziano, J. Melki, C. Hindelang, R. Matyas, P. Rustin, and M. Koenig. 2001. Mouse models for Friedreich ataxia exhibit cardiomyopathy, sensory nerve defect and Fe-S enzyme deficiency followed by intramitochondrial iron deposits. *Nat Genet*. 27:181-6.

Rothe, J., W. Lesslauer, H. Lotscher, Y. Lang, P. Koebel, F. Kontgen, A. Althage, R. Zinkernagel, M. Steinmetz, and H. Bluethmann. 1993. Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by Listeria monocytogenes. *Nature*. 364:798-802.

Rubinsztein, D.C. 2006. The roles of intracellular protein-degradation pathways in neurodegeneration. *Nature*. 443:780-6.

Seil, F.J., M.L. Johnson, and R. Hawkes. 1995. Molecular compartmentation expressed in cerebellar cultures in the absence of neuronal activity and neuron-glia interactions. *J Comp Neurol*. 356:398-407.

Sillitoe, R.V., and A.L. Joyner. 2007. Morphology, molecular codes, and circuitry produce the three-dimensional complexity of the cerebellum. *Annu Rev Cell Dev Biol*. 23:549-77.

Smeyne, R.J., T. Chu, A. Lewin, F. Bian, S. Sanlioglu, C. Kunsch, S.A. Lira, and J. Oberdick. 1995. Local control of granule cell generation by cerebellar Purkinje cells. *Mol Cell Neurosci*. 6:230-51.

Suzuki, K., and Y. Ohsumi. 2007. Molecular machinery of autophagosome formation in yeast, Saccharomyces cerevisiae. *FEBS Lett*. 581:2156-61.

Tai, H.C., and E.M. Schuman. 2008. Ubiquitin, the proteasome and protein degradation in neuronal function and dysfunction. *Nat Rev Neurosci*. 9:826-38.

Tronche, F., C. Kellendonk, O. Kretz, P. Gass, K. Anlag, P.C. Orban, R. Bock, R. Klein, and G. Schutz. 1999. Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat Genet*. 23:99-103.

Ueda, H., S. Abbi, C. Zheng, and J.L. Guan. 2000. Suppression of Pyk2 kinase and cellular activities by FIP200. *J Cell Biol*. 149:423-30.

Wang, V.Y., and H.Y. Zoghbi. 2001. Genetic regulation of cerebellar development. *Nat Rev Neurosci*. 2:484-91.

Wassef, M., C. Sotelo, M. Thomasset, A.C. Granholm, N. Leclerc, J. Rafrafi, and R. Hawkes. 1990. Expression of compartmentation antigen zebrin I in cerebellar transplants. *J Comp Neurol*. 294:223-34.

Wei, H., B. Gan, X. Wu, and J.L. Guan. 2009. Inactivation of FIP200 Leads to Inflammatory Skin Disorder, but Not Tumorigenesis, in Conditional Knock-out Mouse Models. *J Biol Chem*. 284:6004-13.

Winslow, A.R., and D.C. Rubinsztein. 2008. Autophagy in neurodegeneration and development. *Biochim Biophys Acta*. 1782:723-9.

Wong, H.K., M. Fricker, A. Wyttenbach, A. Villunger, E.M. Michalak, A. Strasser, and A.M. Tolkovsky. 2005. Mutually exclusive subsets of BH3-

only proteins are activated by the p53 and c-Jun N-terminal kinase/c-Jun signaling pathways during cortical neuron apoptosis induced by arsenite. *Mol Cell Biol*. 25:8732-47.

Wood, K.A., and R.J. Youle. 1995. The role of free radicals and p53 in neuron apoptosis in vivo. *J Neurosci*. 15:5851-7.

Yuan, J., M. Lipinski, and A. Degterev. 2003. Diversity in the mechanisms of neuronal cell death. *Neuron*. 40:401-13.

Zhuo, L., M. Theis, I. Alvarez-Maya, M. Brenner, K. Willecke, and A. Messing. 2001. hGFAP-cre transgenic mice for manipulation of glial and neuronal function in vivo. *Genesis*. 31:85-94.

CHAPTER 4
CONCLUSIONS AND PROSPECTS

4.1 Conclusions

FIP200 has been shown to play a role in various cellular functions via regulating numerous signaling pathways. My study emphasized the critical role of FIP200 in neuronal homeostasis and survival. Ablation of FIP200 in the neural progenitor cells led to several typical neurophathies, including accumulation of polyubiquitin-containing protein aggregates, progressive axonopathy and spongiform degeneration, as well as increased neuronal cell death. These cellular defects resulted in growth retardation, development of ataxia and paralysis, as well as early death in FIP200 mutant mice. Studies using mice with FIP200 deletion in the Purkinje cell only demonstrated the Purkinje cell autonomous function of FIP200 in the regulation of cerebellar degeneration and ataxia. At the molecular level, the study also addressed the importance of FIP200 in autophagy, particularly the autophagosome formation in CNS. Owed to the intact function of the UPS and the deficiency in autophagosome formation that I found in previous chapters, the results suggested that the deficiency of autophagosome formation is responsible for the accumulation of abnormal protein aggregates in FIP200 mutant mice. Additionally, unhealthy and fragmented mitochondria were found in the Purkinje cells, suggesting that FIP200 is likely involved in mitochondrial fusion and fission machinery to maintain the intact function of mitochondria. The dysfunctional mitochondria may further produce increase cellular levels of ROS to damage the other cellular machineries. More interestingly, the inactivation of TNFR-1 in L7-CKO transgenic mice resulted in a significant

improvement in the Purkinje cell survival and removal of spongiform degeneration. However, the inactivation of TNFR-1 in L7-CKO mutant mice is unable to prevent the Purkinje cells from the formation of protein aggregates and axonal swelling. The results indicated that the death of Purkinje cells and the spongiform degeneration are tightly correlated with the TNF-R1-FIP200 pathway. In contrast, the transgenic mice with the specific deletion of both FIP200 and p53 in the Purkinje cells still showed protein aggregates, loss of the Purkinje cells, spongiform degeneration, and ataxia. The data suggested that the loss of the Purkinje cells is not via a p53-dependent mechanism in FIP200 L7-CKO mice.

4.2 Future Prospects

4.2.1 UPS, autophagy, and protein aggregates

Among various phenotypes found in the FIP200 mutant mice, polyubiquitin aggregates is the key feature that leads to neurodegeneration. It is well known that both the UPS and autophagy are the major cellular protein quality control systems. The deficiency in the UPS or autophagy causes aberrant protein accumulation within the cells. The protein aggregates could be either neurotoxic to cells or neuroprotective to remove the soluble toxic proteins in the cytoplasm. The results amassed from our FIP200 mutant mice clearly showed that protein aggregates generated is because of the deficiency of autophagosome formation but not the altered proteasome catalytic activity. Recent studies have shown that FIP200 forms a functional protein complex with ULKs and Atg13 to initiate autophagosome formation (Hara et al., 2008). This evidence suggests that the protein aggregates found in the FIP200 mutant mice are likely resulting from the defects in the ULK1-Atg13-FIP200

protein complex. However, it remains ambiguous what is the role of the ULK1-Atg13-FIP200 protein complex is *in vivo*, particularly in CNS. Therefore, it will be interesting to study whether the ULK1-Atg13-FIP200 protein complex is involved in neural development, differentiation, and synapse formation by analyzing ULK1-null mice (Kundu et al., 2008) and Atg13-null transgenic mice (if available).

Another question for the ULK1-Atg13-FIP200 protein complex is the potential different physiological consequences between the deficiency of autophagosome initiation caused by deletion of FIP200 (or ULK1, Atg13) and Atg5/7 null-mediated autophagy defects. Recent reports have revealed that the conditional KO of Atg5 or Atg7 in CNS led to the aggregation of polyubiquitinated proteins resulting neurodegeneration (Hara et al., 2006; Komatsu et al., 2006). The results collected from Atg7-null transgenic mice suggest that the death of the Purkinje cell is via apoptosis. In contrast, the loss of the Purkinje cells in FIP200 mutant mice is TUNEL negative, and, furthermore, it is independent of p53-mediate apoptosis. These observations also make it interesting to investigate the causes for the loss of the Purkinje cells in FIP200 mutant mice. As mentioned in the introduction, the aggregation of polyubiquitin-containing proteins leads to loss of their normal functions that could lead to cell death. If the death of the Purkinje cells is owed to protein aggregates, the content of the aggregates could be different between FIP200 CKO and Atg5/7 CKO mice. It is possible to identify the contents in the protein aggregates like the case that the scientists finally targeted TDP-43 is the major aggregated protein in the inclusion bodies of ALS patients (Neumann et al., 2006). Therefore, identification of the contents in protein

aggregates from FIP200 mutant mice may reveal the role of ULK1-Atg13-FIP200 functions *in vivo*.

Although my data suggest that the protein aggregates is not caused by impaired proteasome catalytic activity in FIP200 CKO mice, it is still possible that FIP200 could regulate the UPS by functioning as a proteasomal targeting protein. A recent study has revealed that a group of proteins, such as UFD2, RAD23, DSK2 and RPN10, escort the ubiquitinated proteins to proteasome for ultimate degradation (Richly et al., 2005). Preliminary data from others in our lab showed that FIP200 can associate with both ubiquitin and several subunits of 19s proteasome. Therefore, it is also interesting to study whether FIP200 is an ubiquitin binding protein to transfer ubiquitinylated substrates to proteasome.

4.2.1 Mitochondria, ROS and spongiform degeneration

Spongiform degeneration is a rare neuropathic feature. It has been proposed that dysfunctional proteasome and altered ubiquitination process may contribute to the phenotype. However, the results collected from proteasome catalytic activities analysis showed that 20S proteasome subunits had slightly increased catalytic activities in FIP200 CKO mice. Therefore, the malfunction of the UPS is not the case to cause spongiform degeneration in the FIP200 mutant mice. Indeed, the accumulation of polyubiquitin-containing protein can affect a number of cellular pathways by increasing cellular stress. According to a recent report, when the cell is exposed to mild oxidative stress (Zeevalk and Bernard, 2005) it is often found to exhibit the elevated proteasome activity. The increase in catalytic activity of the proteasome was proposed to be the response to facilitate the removal of

protein substrates. One major source of cellular stress is the ROS (Berlett and Stadtman, 1997; Klein and Ackerman, 2003). ROS are primarily produced from the mitochondrial electron transport chain (ETC). The ETC has been identified as one of the cellular generators of ROS, including superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl free radical (·OH) (Boveris et al., 1972; Chance et al., 1979; Loschen et al., 1971). The cellular level of ROS is also controlled by cellular antioxidant systems (Barrientos and Moraes, 1999; Chen et al., 2003; Nicholls, 2002; Nohl et al., 2003).

Although low levels of ROS can function as an intracellular signal (Aslan and Ozben, 2003; Finkel, 2001), high levels of ROS change protein conformation, alter enzyme activity including proteasome (Carrard et al., 2002), and increase abnormal ubiquitinated protein aggregation (Adamo et al., 1999; Ardley et al., 2004; Biasini et al., 2004; Rideout et al., 2001; Rideout and Stefanis, 2002). Furthermore, the impaired mitochondria can lead to an increase of ROS and release of mitochondrial components into the cytoplasm, which trigger cell death (Chinopoulos and Adam-Vizi, 2001; Chinopoulos et al., 1999; Zhang et al., 2001). The mitochondria-mediated cell death can be intrinsic or extrinsic (Donovan and Cotter, 2004; Li et al., 2004; Marshansky et al., 2001; Peruzzi et al., 2001). Interestingly, recent studies indicate that a cross-talk mechanism between the UPS and mitochondria plays a role in maintain both system activities (Carrard et al., 2002; Ding and Keller, 2001; Li et al., 2003; Papa et al., 2007; Rinaldi et al., 2004; Shamoto-Nagai et al., 2003; Sullivan et al., 2004; Zeevalk and Bernard, 2005) as well as in cell death (Papa and Rockwell, 2008). The UPS may be also crucial for the the maintenance of mitochondrial function and health by regulating the mitochondrial fusion

and fission machinery (Altmann and Westermann, 2005; Durr et al., 2006; Fritz et al., 2003; Nakamura and Hirose, 2008; Nakamura et al., 2006).

The TEM results amassed from the Purkinje cells of Nestin-CKO mice showed that abnormal fragmented mitochondria and the IHC data gathered from L7-CKO exhibited an increase of cytochrome c in the Purkinje cells. The data suggested that the function of mitochondria may be regulated by FIP200. Although it remains unclear how FIP200 involves in the mitochondrial machinery, the studies from two knockout models, manganese superoxide dismutase (Sod2) and NF-E2-related factor (Nrf2) in oxidative stress signaling, associated with mitochondrial dysfunction and spongiform degeneration (Hubbs et al., 2007; Melov et al., 1998; Pederzolli et al., 2007; Zhang et al., 2001). The results suggest the important role of ROS regulation in the brain. In addition, mutation of ETC complex IV subunit VIa causes deficiency in mitochondrial function, leading to spongiform change in fruit flies (Liu et al., 2007). These results indicate that malfunction of mitochondria and an increase of ROS can cause vacuolization and cell death in the brain. Therefore, it will be interesting to study the role of FIP200 in several mitochondrial functions, including ETC, fusion and fission machinery, as well as cellular antioxidant systems.

In addition, studies have shown that oxidative-stress-induced spongiform pathology can be reduced by the treatment of antioxidants (Gomi et al., 1994; Hinerfeld et al., 2004; Jiang et al., 2006; Pederzolli et al., 2007). The antioxidant treatment decreases cell death in *Zitter* rats (Gomi et al., 1994), *SOD2*$^{-/-}$ mice (Hinerfeld et al., 2004), and cells exposed to N-acetyl-l-aspartate (NAA) (Pederzolli et al., 2007). High levels of ROS can also promote protein aggregation (Carrard et al., 2002; Dear et al., 2007; Ross and Poirier,

2004; Shamoto-Nagai et al., 2003) and trigger apoptosis signaling in neurons, resulting in spongiform degeneration and massive cell death. Similar to Prion functioning as a potential antioxidant by binding copper, FIP200 could play a role in the regulation of ROS.

4.2.3 TNFR-1-FIP200 pathway in neurogenesis and neurodegeneration

The most intriguing finding in my study is that loss of the Purkinje cells, spongiform degeneration, and incoordinated movement of L7-CKO mice were significantly rescued by inaction of TNFR-1. Moreover, cytochrome c release in L7-CKO mice was also reduced upon inactivation of TNFR-1. These data strongly suggest that altered TNF-R1 pathway is responsible for the neurophathic features found in L7-CKO cerebellum. As mentioned in the last section, release of cytochrome c is resulting from dysfunctional mitochondria under oxidative stress. Hence, it will be interesting to study the mechanism by which TNFR-1-FIP200 pathway regulates cell survival in cerebellum.

Neurotrophic factors play an important role in many cellular functions of neurons, including cell survival (Hempstead, 2006), axon outgrowth and guidance (Reichardt, 2006), and cell growth (Allen and Dawbarn, 2006). A recent study has revealed that DR6, a TNF superfamily members, triggers neurodegeneration through different caspases (Nikolaev et al., 2009). The result implies that the activation TNFR-1 may also regulate neuronal survival. For example, in the normal Purkinje cells, the activation of TNFR-1 can recruit FIP200, resulting in phosphorylation of JNK and cell survival. However, in the L7-CKO mice, the activation of TNFR-1 may initiate apoptosis via caspases cascade.

Numerous studies have shown that TNFα is the primary factor of immune response in CNS (Nguyen et al., 2002; Turrin and Rivest, 2006). TNFα regulates various cellular effects by activating two distinct receptors, TNFR-1 mainly for neuronal death and TNF-R2 for neuroprotection (Bernardino et al., 2005; Fontaine et al., 2002; Marchetti et al., 2004; Yang et al., 2002). It has been shown that both neural stem cell (NSC) and progenitor cells express TNFα (Iosif et al., 2006; Klassen et al., 2003) as well as the receptors (Ben-Hur et al., 2003; Cacci et al., 2005; Iosif et al., 2006; Sheng et al., 2005; Widera et al., 2006). Several recent studies emphasize the essential role of TNFα in neurogenesis. For example, an increase in proliferation is found at the subventricular zone in the TNFα-treated rats (Wu et al., 2000) and *in vivo* infusion of a neutralizing antibody against TNFα reduce the number of neuroblasts in striatum and hippocampus (Heldmann et al., 2005). Interestingly, a study using TNFα receptors null mice has demonstrated that in neural progenitor cells TNFR-1 acts as a suppressor for proliferation but TNFR-2 functions as a promotor for their survival (Iosif et al., 2006). Nuclear factor B and cyclin D1 have been shown to regulate the TNFα-induced proliferation of NSCs *in vitro* (Widera et al., 2006). Furthermore, the TNFα-activated SAPK/JNK signaling pathway is also important in regulation of neuronal cell proliferation and death (Bernardino et al., 2008). Taken collectively, the recent results suggest an essential role of TNFα in neurogenesis.

Although the preliminary results are not included in the study, TNFR-1 null mice with FIP200 deletion in neural progenitor cells (*FIP200*$^{flox/flox}$, *TNFR-1*$^{-/-}$; nestin-Cre) were born in Mendelian ratio but died within 72 hr, which is even earlier than CKO mice. I found that *FIP200*$^{flox/flox}$, *TNFR-1*$^{-/-}$; nestin-Cre mouse showed increased apoptosis in the cerebellum and

hippocampus where neurogenesis occurs compared to CKO mice. The results suggest that TNFR-1-FIP200 pathway may be critical for CNS development. It will be interesting to investigate the synergistic effect of TNFR-1 and FIP200 on neurogenesis and neurodegeneration. The results will reveal the importance of TNF-R1-FIP200 pathway in both mitotic and post-mitotic neurons. In addition, TNFR-2 expresses in the NSCs and its downstream molecules, like TRAF-2, can interact with FIP200. It will be interesting to investigate the mechanism by which FIP200 regulates NSC survival and proliferation through TNFR-2.

REFERENCES

Adamo, A.M., L.A. Pasquini, M.B. Moreno, P.I. Oteiza, E.F. Soto, and J.M. Pasquini. 1999. Effect of oxidant systems on the ubiquitylation of proteins in the central nervous system. *J Neurosci Res*. 55:523-31.

Allen, S.J., and D. Dawbarn. 2006. Clinical relevance of the neurotrophins and their receptors. *Clin Sci (Lond)*. 110:175-91.

Altmann, K., and B. Westermann. 2005. Role of essential genes in mitochondrial morphogenesis in Saccharomyces cerevisiae. *Mol Biol Cell*. 16:5410-7.

Ardley, H.C., G.B. Scott, S.A. Rose, N.G. Tan, and P.A. Robinson. 2004. UCH-L1 aggresome formation in response to proteasome impairment indicates a role in inclusion formation in Parkinson's disease. *J Neurochem*. 90:379-91.

Aslan, M., and T. Ozben. 2003. Oxidants in receptor tyrosine kinase signal transduction pathways. *Antioxid Redox Signal*. 5:781-8.

Barrientos, A., and C.T. Moraes. 1999. Titrating the effects of mitochondrial complex I impairment in the cell physiology. *J Biol Chem*. 274:16188-97.

Ben-Hur, T., O. Ben-Menachem, V. Furer, O. Einstein, R. Mizrachi-Kol, and N. Grigoriadis. 2003. Effects of proinflammatory cytokines on the growth, fate, and motility of multipotential neural precursor cells. *Mol Cell Neurosci*. 24:623-31.

Berlett, B.S., and E.R. Stadtman. 1997. Protein oxidation in aging, disease, and oxidative stress. *J Biol Chem*. 272:20313-6.

Bernardino, L., F. Agasse, B. Silva, R. Ferreira, S. Grade, and J.O. Malva. 2008. Tumor necrosis factor-alpha modulates survival, proliferation, and

neuronal differentiation in neonatal subventricular zone cell cultures. *Stem Cells*. 26:2361-71.

Bernardino, L., S. Xapelli, A.P. Silva, B. Jakobsen, F.R. Poulsen, C.R. Oliveira, A. Vezzani, J.O. Malva, and J. Zimmer. 2005. Modulator effects of interleukin-1beta and tumor necrosis factor-alpha on AMPA-induced excitotoxicity in mouse organotypic hippocampal slice cultures. *J Neurosci*. 25:6734-44.

Biasini, E., L. Fioriti, I. Ceglia, R. Invernizzi, A. Bertoli, R. Chiesa, and G. Forloni. 2004. Proteasome inhibition and aggregation in Parkinson's disease: a comparative study in untransfected and transfected cells. *J Neurochem*. 88:545-53.

Boveris, A., N. Oshino, and B. Chance. 1972. The cellular production of hydrogen peroxide. *Biochem J*. 128:617-30.

Cacci, E., J.H. Claasen, and Z. Kokaia. 2005. Microglia-derived tumor necrosis factor-alpha exaggerates death of newborn hippocampal progenitor cells in vitro. *J Neurosci Res*. 80:789-97.

Carrard, G., A.L. Bulteau, I. Petropoulos, and B. Friguet. 2002. Impairment of proteasome structure and function in aging. *Int J Biochem Cell Biol*. 34:1461-74.

Chance, B., H. Sies, and A. Boveris. 1979. Hydroperoxide metabolism in mammalian organs. *Physiol Rev*. 59:527-605.

Chen, Q., E.J. Vazquez, S. Moghaddas, C.L. Hoppel, and E.J. Lesnefsky. 2003. Production of reactive oxygen species by mitochondria: central role of complex III. *J Biol Chem*. 278:36027-31.

Chinopoulos, C., and V. Adam-Vizi. 2001. Mitochondria deficient in complex I activity are depolarized by hydrogen peroxide in nerve terminals: relevance to Parkinson's disease. *J Neurochem*. 76:302-6.

Chinopoulos, C., L. Tretter, and V. Adam-Vizi. 1999. Depolarization of in situ mitochondria due to hydrogen peroxide-induced oxidative stress in nerve terminals: inhibition of alpha-ketoglutarate dehydrogenase. *J Neurochem*. 73:220-8.

Dear, D.V., D.S. Young, J. Kazlauskaite, F. Meersman, D. Oxley, J. Webster, T.J. Pinheiro, A.C. Gill, I. Bronstein, and C.R. Lowe. 2007. Effects of post-translational modifications on prion protein aggregation and the propagation of scrapie-like characteristics in vitro. *Biochim Biophys Acta*. 1774:792-802.

Ding, Q., and J.N. Keller. 2001. Proteasome inhibition in oxidative stress neurotoxicity: implications for heat shock proteins. *J Neurochem*. 77:1010-7.

Donovan, M., and T.G. Cotter. 2004. Control of mitochondrial integrity by Bcl-2 family members and caspase-independent cell death. *Biochim Biophys Acta*. 1644:133-47.

Durr, M., M. Escobar-Henriques, S. Merz, S. Geimer, T. Langer, and B. Westermann. 2006. Nonredundant roles of mitochondria-associated F-box proteins Mfb1 and Mdm30 in maintenance of mitochondrial morphology in yeast. *Mol Biol Cell*. 17:3745-55.

Finkel, T. 2001. Reactive oxygen species and signal transduction. *IUBMB Life*. 52:3-6.

Fontaine, V., S. Mohand-Said, N. Hanoteau, C. Fuchs, K. Pfizenmaier, and U. Eisel. 2002. Neurodegenerative and neuroprotective effects of tumor

Necrosis factor (TNF) in retinal ischemia: opposite roles of TNF receptor 1 and TNF receptor 2. *J Neurosci*. 22:RC216.

Fritz, S., N. Weinbach, and B. Westermann. 2003. Mdm30 is an F-box protein required for maintenance of fusion-competent mitochondria in yeast. *Mol Biol Cell*. 14:2303-13.

Gomi, H., I. Ueno, and K. Yamanouchi. 1994. Antioxidant enzymes in the brain of zitter rats: abnormal metabolism of oxygen species and its relevance to pathogenic changes in the brain of zitter rats with genetic spongiform encephalopathy. *Brain Res*. 653:66-72.

Hara, T., K. Nakamura, M. Matsui, A. Yamamoto, Y. Nakahara, R. Suzuki-Migishima, M. Yokoyama, K. Mishima, I. Saito, H. Okano, and N. Mizushima. 2006. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature*. 441:885-9.

Hara, T., A. Takamura, C. Kishi, S. Iemura, T. Natsume, J.L. Guan, and N. Mizushima. 2008. FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells. *J Cell Biol*. 181:497-510.

Heldmann, U., P. Thored, J.H. Claasen, A. Arvidsson, Z. Kokaia, and O. Lindvall. 2005. TNF-alpha antibody infusion impairs survival of stroke-generated neuroblasts in adult rat brain. *Exp Neurol*. 196:204-8.

Hempstead, B.L. 2006. Dissecting the diverse actions of pro- and mature neurotrophins. *Curr Alzheimer Res*. 3:19-24.

Hinerfeld, D., M.D. Traini, R.P. Weinberger, B. Cochran, S.R. Doctrow, J. Harry, and S. Melov. 2004. Endogenous mitochondrial oxidative stress: neurodegeneration, proteomic analysis, specific respiratory chain defects, and efficacious antioxidant therapy in superoxide dismutase 2 null mice. *J Neurochem*. 88:657-67.

Hubbs, A.F., S.A. Benkovic, D.B. Miller, J.P. O'Callaghan, L. Battelli, D. Schwegler-Berry, and Q. Ma. 2007. Vacuolar leukoencephalopathy with widespread astrogliosis in mice lacking transcription factor Nrf2. *Am J Pathol*. 170:2068-76.

Iosif, R.E., C.T. Ekdahl, H. Ahlenius, C.J. Pronk, S. Bonde, Z. Kokaia, S.E. Jacobsen, and O. Lindvall. 2006. Tumor necrosis factor receptor 1 is a negative regulator of progenitor proliferation in adult hippocampal neurogenesis. *J Neurosci*. 26:9703-12.

Jiang, Y., V.L. Scofield, M. Yan, W. Qiang, N. Liu, A.J. Reid, W.S. Lynn, and P.K. Wong. 2006. Retrovirus-induced oxidative stress with neuroimmunodegeneration is suppressed by antioxidant treatment with a refined monosodium alpha-luminol (Galavit). *J Virol*. 80:4557-69.

Klassen, H.J., K.L. Imfeld, Kirov, II, L. Tai, F.H. Gage, M.J. Young, and M.A. Berman. 2003. Expression of cytokines by multipotent neural progenitor cells. *Cytokine*. 22:101-6.

Klein, J.A., and S.L. Ackerman. 2003. Oxidative stress, cell cycle, and neurodegeneration. *J Clin Invest*. 111:785-93.

Komatsu, M., S. Waguri, T. Chiba, S. Murata, J. Iwata, I. Tanida, T. Ueno, M. Koike, Y. Uchiyama, E. Kominami, and K. Tanaka. 2006. Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature*. 441:880-4.

Kundu, M., T. Lindsten, C.Y. Yang, J. Wu, F. Zhao, J. Zhang, M.A. Selak, P.A. Ney, and C.B. Thompson. 2008. Ulk1 plays a critical role in the autophagic clearance of mitochondria and ribosomes during reticulocyte maturation. *Blood*. 112:1493-502.

Li, Q., A.K. Ching, B.C. Chan, S.K. Chow, P.L. Lim, T.C. Ho, W.K. Ip, C.K. Wong, C.W. Lam, K.K. Lee, J.Y. Chan, and Y.L. Chui. 2004. A death receptor-associated anti-apoptotic protein, BRE, inhibits mitochondrial apoptotic pathway. *J Biol Chem*. 279:52106-16.

Li, Y.P., Y. Chen, A.S. Li, and M.B. Reid. 2003. Hydrogen peroxide stimulates ubiquitin-conjugating activity and expression of genes for specific E2 and E3 proteins in skeletal muscle myotubes. *Am J Physiol Cell Physiol*. 285:C806-12.

Liu, W., R. Gnanasambandam, J. Benjamin, G. Kaur, P.B. Getman, A.J. Siegel, R.D. Shortridge, and S. Singh. 2007. Mutations in cytochrome c oxidase subunit VIa cause neurodegeneration and motor dysfunction in Drosophila. *Genetics*. 176:937-46.

Loschen, G., L. Flohe, and B. Chance. 1971. Respiratory chain linked H(2)O(2) production in pigeon heart mitochondria. *FEBS Lett*. 18:261-264.

Marchetti, L., M. Klein, K. Schlett, K. Pfizenmaier, and U.L. Eisel. 2004. Tumor necrosis factor (TNF)-mediated neuroprotection against glutamate-induced excitotoxicity is enhanced by N-methyl-D-aspartate receptor activation. Essential role of a TNF receptor 2-mediated phosphatidylinositol 3-kinase-dependent NF-kappa B pathway. *J Biol Chem*. 279:32869-81.

Marshansky, V., X. Wang, R. Bertrand, H. Luo, W. Duguid, G. Chinnadurai, N. Kanaan, M.D. Vu, and J. Wu. 2001. Proteasomes modulate balance among proapoptotic and antiapoptotic Bcl-2 family members and compromise functioning of the electron transport chain in leukemic cells. *J Immunol*. 166:3130-42.

Melov, S., J.A. Schneider, B.J. Day, D. Hinerfeld, P. Coskun, S.S. Mirra, J.D. Crapo, and D.C. Wallace. 1998. A novel neurological phenotype in mice lacking mitochondrial manganese superoxide dismutase. *Nat Genet*. 18:159-63.

Nakamura, N., and S. Hirose. 2008. Regulation of mitochondrial morphology by USP30, a deubiquitinating enzyme present in the mitochondrial outer membrane. *Mol Biol Cell*. 19:1903-11.

Nakamura, N., Y. Kimura, M. Tokuda, S. Honda, and S. Hirose. 2006. MARCH-V is a novel mitofusin 2- and Drp1-binding protein able to change mitochondrial morphology. *EMBO Rep*. 7:1019-22.

Neumann, M., D.M. Sampathu, L.K. Kwong, A.C. Truax, M.C. Micsenyi, T.T. Chou, J. Bruce, T. Schuck, M. Grossman, C.M. Clark, L.F. McCluskey, B.L. Miller, E. Masliah, I.R. Mackenzie, H. Feldman, W. Feiden, H.A. Kretzschmar, J.Q. Trojanowski, and V.M. Lee. 2006. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science*. 314:130-3.

Nguyen, M.D., J.P. Julien, and S. Rivest. 2002. Innate immunity: the missing link in neuroprotection and neurodegeneration? *Nat Rev Neurosci*. 3:216-27.

Nicholls, D.G. 2002. Mitochondrial function and dysfunction in the cell: its relevance to aging and aging-related disease. *Int J Biochem Cell Biol*. 34:1372-81.

Nikolaev, A., T. McLaughlin, D.D. O'Leary, and M. Tessier-Lavigne. 2009. APP binds DR6 to trigger axon pruning and neuron death via distinct caspases. *Nature*. 457:981-9.

Nohl, H., L. Gille, A. Kozlov, and K. Staniek. 2003. Are mitochondria a spontaneous and permanent source of reactive oxygen species? *Redox Rep*. 8:135-41.

Papa, L., E. Gomes, and P. Rockwell. 2007. Reactive oxygen species induced by proteasome inhibition in neuronal cells mediate mitochondrial dysfunction and a caspase-independent cell death. *Apoptosis*. 12:1389-405.

Papa, L., and P. Rockwell. 2008. Persistent mitochondrial dysfunction and oxidative stress hinder neuronal cell recovery from reversible proteasome inhibition. *Apoptosis*. 13:588-99.

Pederzolli, C.D., C.P. Mescka, F. Scapin, F.J. Rockenbach, A.M. Sgaravatti, M.B. Sgarbi, A.T. Wyse, C.M. Wannmacher, M. Wajner, and C.S. Dutra-Filho. 2007. N-acetylaspartic acid promotes oxidative stress in cerebral cortex of rats. *Int J Dev Neurosci*. 25:317-24.

Peruzzi, F., M. Prisco, A. Morrione, B. Valentinis, and R. Baserga. 2001. Anti-apoptotic signaling of the insulin-like growth factor-I receptor through mitochondrial translocation of c-Raf and Nedd4. *J Biol Chem*. 276:25990-6.

Reichardt, L.F. 2006. Neurotrophin-regulated signalling pathways. *Philos Trans R Soc Lond B Biol Sci*. 361:1545-64.

Richly, H., M. Rape, S. Braun, S. Rumpf, C. Hoege, and S. Jentsch. 2005. A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. *Cell*. 120:73-84.

Rideout, H.J., K.E. Larsen, D. Sulzer, and L. Stefanis. 2001. Proteasomal inhibition leads to formation of ubiquitin/alpha-synuclein-immunoreactive inclusions in PC12 cells. *J Neurochem*. 78:899-908.

Rideout, H.J., and L. Stefanis. 2002. Proteasomal inhibition-induced inclusion formation and death in cortical neurons require transcription and ubiquitination. *Mol Cell Neurosci*. 21:223-38.

Rinaldi, T., E. Pick, A. Gambadoro, S. Zilli, V. Maytal-Kivity, L. Frontali, and M.H. Glickman. 2004. Participation of the proteasomal lid subunit Rpn11 in mitochondrial morphology and function is mapped to a distinct C-terminal domain. *Biochem J*. 381:275-85.

Ross, C.A., and M.A. Poirier. 2004. Protein aggregation and neurodegenerative disease. *Nat Med*. 10 Suppl:S10-7.

Shamoto-Nagai, M., W. Maruyama, Y. Kato, K. Isobe, M. Tanaka, M. Naoi, and T. Osawa. 2003. An inhibitor of mitochondrial complex I, rotenone, inactivates proteasome by oxidative modification and induces aggregation of oxidized proteins in SH-SY5Y cells. *J Neurosci Res*. 74:589-97.

Sheng, W.S., S. Hu, H.T. Ni, T.N. Rowen, J.R. Lokensgard, and P.K. Peterson. 2005. TNF-alpha-induced chemokine production and apoptosis in human neural precursor cells. *J Leukoc Biol*. 78:1233-41.

Sullivan, P.G., N.B. Dragicevic, J.H. Deng, Y. Bai, E. Dimayuga, Q. Ding, Q. Chen, A.J. Bruce-Keller, and J.N. Keller. 2004. Proteasome inhibition alters neural mitochondrial homeostasis and mitochondria turnover. *J Biol Chem*. 279:20699-707.

Turrin, N.P., and S. Rivest. 2006. Tumor necrosis factor alpha but not interleukin 1 beta mediates neuroprotection in response to acute nitric oxide excitotoxicity. *J Neurosci*. 26:143-51.

Widera, D., I. Mikenberg, M. Elvers, C. Kaltschmidt, and B. Kaltschmidt. 2006. Tumor necrosis factor alpha triggers proliferation of adult neural stem cells via IKK/NF-kappaB signaling. *BMC Neurosci*. 7:64.

Wu, J.P., J.S. Kuo, Y.L. Liu, and S.F. Tzeng. 2000. Tumor necrosis factor-alpha modulates the proliferation of neural progenitors in the subventricular/ventricular zone of adult rat brain. *Neurosci Lett*. 292:203-6.

Yang, L., K. Lindholm, Y. Konishi, R. Li, and Y. Shen. 2002. Target depletion of distinct tumor necrosis factor receptor subtypes reveals hippocampal neuron death and survival through different signal transduction pathways. *J Neurosci*. 22:3025-32.

Zeevalk, G.D., and L.P. Bernard. 2005. Energy status, ubiquitin proteasomal function, and oxidative stress during chronic and acute complex I inhibition with rotenone in mesencephalic cultures. *Antioxid Redox Signal*. 7:662-72.

Zhang, J.G., M.A. Tirmenstein, F.A. Nicholls-Grzemski, and M.W. Fariss. 2001. Mitochondrial electron transport inhibitors cause lipid peroxidation-dependent and -independent cell death: protective role of antioxidants. *Arch Biochem Biophys*. 393:87-96.